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CHEMICAL STUDIES OF ISOLATED MYOFIBRILS *IN VITRO*

I. THE ROLE OF MAGNESIUM, CALCIUM AND NITROGEN-PHOSPHORUS PARTITION IN CONTRACTION INDUCED WITH ADENOSINETRIPHOSPHATE

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The study of intracellular structure and chemistry has been a part of the search for an understanding of the mechanisms of cellular function. The demonstration that one such function, namely muscular contraction, is accompanied by the splitting of phosphate from ATP[2] suggested the important role of this compound [18]. The "high-energy" phosphate bonds have been considered the probable chemical source of energy for muscular contraction and for other types of cellular function [15]. ATP is present in large amounts in muscle [16, 7]. Theoretically, the energy is made available by the splitting of these phosphate bonds either before, during or after the contraction process. Little is known of the mechanism by which this chemical energy is converted into mechanical work or tension.

It seemed probable that additional information could be obtained by study of the reaction between the myofibril, which is the contractile element of muscle, and ATP. This type of study was made possible by development of methods for isolation of myofibrils from skeletal and cardiac muscle using either tryptic digestion at 0° C [20, 21] or the mechanical forces of a colloid mill [2] to facilitate rupture of the muscle cells with liberation of individual myofibrils. Microscopic observation revealed that addition of ATP to a suspension of these purified myofibrils resulted in instantaneous contraction with conversion of myofibrils into small round or oval masses, the degree of contraction being related to the amount of ATP added [20, 21].

Electron microscopic studies [2] comparing the structure of myofibrils in the uncontracted state with those in progressive stages of ATP-induced contraction revealed changes similar to those noted by other workers using *in*

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[2] Abbreviations used: ATP – adenosinetriphosphate; ADP – adenosinediphosphate; AMP – adenylic acid; ATPase – adenosinetriphosphatase.

vivo stimulation [4, 11]. These changes included a marked shift in the localization of the cross bands during contraction, indicating a migration or redistribution of material within the myofibril. Measurements of the individual filaments of which the myofibrils were composed revealed an increase in their mean diameter in the contracted state.

Initial chemical studies [1] of this contractile reaction differed from those of other workers in which excessive amounts of ATP were used and in which many minutes or hours were required for ATP-splitting [13, 19]. This splitting over prolonged intervals of time would appear to have little significance in studies designed to analyze the chemical events of an almost instantaneous contraction. In our studies only the amount of ATP necessary to insure complete contraction of the myofibrils was used. The added ATP was converted to ADP by an ATPase which was intimately associated with or a part of the myofibril. No "large-scale" permanent binding of the nucleotides occurred, since 95 to 98 per cent of the added ATP was recovered after contraction as ADP. Myokinase was found to be unessential for contraction [1].

On the theoretical grounds it seemed probable that some chemical change occurred in the myofibril during the process of contraction. The only change detected in the added ATP was that resulting from the ATPase action of the myofibrils. Furthermore, it had already been shown that the ADP resulting from this ATPase action was not permanently bound to the contracted myofibril [1]. The present study was undertaken to determine the fate of the phosphate group split from the ATP and the relationship of this ATPase action to the contractile event. Whole muscle contains several ATPases which have different pH optima and activating ions, including calcium and magnesium. Under certain conditions these ions are antagonistic [10, 12, 21]. In the present study, data were obtained on the effects of calcium and magnesium on both ATP-induced contraction and ATP-splitting by purified myofibrils.

METHODS

Isolation and purification of myofibrils.—Rabbit skeletal muscle, cut into fragments measuring less than one centimeter in greatest dimension, was suspended in 0.15 *M* KCl solution. The amount of muscle varied from 50 to 400 grams. The most satisfactory results were obtained with a 3 to 1 ratio of KCl solution to muscle. The fragments of muscle and solution were placed in an Eppenbach Colloid Mill (Type QV-7-1) equipped with a water cooled jacket and a recirculating device. The optimal distance between rotor and stator and the speed of operation were determined by trial and error. The mill was run for periods of 15 seconds with the recirculating device

open. The above proportions of muscle and solution gave a very viscous, thick suspension. Milling was continued until microscopic examination revealed that more than 50 per cent of the muscle cells had disintegrated, releasing the myofibrils. This was usually accomplished in less than one minute of operation.

All subsequent procedures were carried out in a cold room at 0°C. The myofibrils were first purified by differential centrifugation and then washed in 0.15 *M* KCl. This was followed by washings with this same solution containing either 5 or 10 parts per 100 of succinate buffer solution (pH 6.1, 0.16 *M*) and 0.5 gm $MgCl_2$ per liter. This will subsequently be referred to as KCl-succinate-Mg solution. In certain experiments when it was desirable to remove traces of myokinase activity, it was necessary to wash first with a solution in which a potassium phosphate-citric acid buffer solution (pH 8.0, μ 0.15) was substituted for the succinate buffer. When this solution was used, final washings in the 0.15 *M* KCl-succinate-Mg solution were continued until the supernatants were free of phosphorus.

Addition of ATP to a suspension of these myofibrils resulted in instantaneous contraction. Only the amount of ATP required to produce complete contraction of a given quantity of myofibrils was used. This was determined by microscopic observation of a series of tubes each containing an equal amount of myofibril suspension but to which different amounts of ATP were added. The amount of ATP added to the first tube in which the myofibrils were completely contracted was used in subsequent experiments.

Preparation of "magnesium-depleted" myofibrils.—In the preparation of "magnesium-depleted" myofibrils the initial steps were the same. The suspension of myofibrils was placed in a dialyzing sac and dialyzed for 3 to 5 days against a continuous flow of 0.15 *M* KCl solution containing 10 parts per 100 of potassium phosphate-citric acid buffer solution (pH 8.0, μ 0.15). They were then washed, dialyzed for 1 to 3 days and suspended in 0.15 *M* KCl containing 5 to 10 parts of succinate buffer solution (pH 6.1, 0.15 *M*). This will be referred to subsequently as KCl-succinate solution. Analyses were made of the magnesium content of these myofibrils by the method of Eichelberger and Bibler [5]. Dry, dialyzed myofibrils had a magnesium content of 8.9 to 10.3 micrograms per 100 mgs compared to 113 to 126 micrograms per 100 mgs in dry fat free muscle.

Although these "magnesium depleted" myofibrils retained their structure, addition of ATP resulted in contraction only when magnesium ions were added prior to or at the same time as ATP.

Analytical methods.—The phosphorus and nitrogen contents of uncontracted and contracted myofibrils were determined. After centrifugation, one half of the myofibrils were washed with buffer solution alone; the other half with buffer solution followed by ethyl alcohol and ether. After these samples were dried at 106°C for 24 to 72 hours, 25 or 50 mgs of each were wet-ashed with H_2SO_4 and 30 per cent hydrogen peroxide. The total phosphorus was determined by the method of Fiske and Subbarow [6]; the total nitrogen by direct nesslerization [14].

Supernatant fluids, removed from the myofibrils were analyzed for inorganic phosphorus by Lowry's [17] method and by that of Fiske and Subbarow [6]. Results of the two methods were nearly identical, and for most experiments the latter was used. Other portions of supernatant fluids were wet-ashed, and the total phosphorus and nitrogen were determined in the manner described above.

RESULTS

Changes in phosphorus and nitrogen

Phosphorus content of supernatants. — To detect permanent binding to the myofibril of the terminal phosphate group split off from ATP during contraction, the supernatant of the contracted myofibrils and control solutions were analyzed for total and inorganic phosphorus. To a suspension containing approximately 500 mgs of myofibrils (50 mgs dry weight) in 20 mls of KCl-succinate-Mg solution, 17.5 mgs of the sodium salt of ATP[1] in 5 ml of the above solution were added to give a final concentration of 0.7 mg of sodium ATP per ml of suspension or a theoretical total phosphorus of 87 micrograms/ml. Complete contraction occurred immediately at room temperature. The suspension was centrifuged and the supernatant removed and filtered through Buchner type fritted glass filters of fine grade. This supernatant was analyzed for inorganic and total phosphorus. Control analyses were made on the diluted ATP stock solution, the supernatant of the uncontracted myofibrils, and the supernatant which was first removed from uncontracted myofibrils and to which ATP was then added. The results of five consecutive experiments are shown in Table I. In each case, the super-

TABLE I

Total phosphorus and inorganic phosphorus in supernatants of contracted myofibrils and controls.

	Phosphorus, micrograms per ml									
	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Experiment 5	
	Total	Inorg.	Total	Inorg.	Total	Inorg.	Total	Inorg.	Total	Inorg.
1. Supernatant of uncontracted myofibrils . . .	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2. ATP stock solution . .	66.1	4.0	61.8	3.5	53.9	3.1	63.0	4.0	60.2	4.3
3. ATP added to supernatant removed from uncontracted myofibrils	66.1	3.9	61.6	3.5	54.2	3.1	63.9	3.0	60.9	4.3
4. Supernatant of myofibrils contracted with ATP	70.0	19.0	63.8	22.9	57.8	18.7	65.5	22.4	62.5	15.0

[1] The ATP was obtained from the Sigma Co., St. Louis, as the barium salt and was converted to the sodium salt before use.

natants removed from the uncontracted myofibrils were free of phosphorus (line 1 of Table I). The ATP stock solution which was diluted by addition to a volume of KCl-succinate-Mg solution equal to the volume of myofibril suspension used in the contracting system contained 3.1 to 4.3 micrograms of inorganic phosphorus (line 2 of Table I). Supernatant was removed from uncontracted myofibrils. ATP was added to a volume of this supernatant equal to that of the myofibril suspension used in the contracting system. There was no significant change in either total or inorganic phosphorus. (Compare lines 3 and 2 of Table I.) The addition of ATP directly to the myofibrils resulted in instantaneous contraction. The supernatant of these contracted myofibrils showed an increase in inorganic phosphorus, but no decrease in total phosphorus. (Compare lines 4 with 2 and 3 of Table I.) In every experiment, there was a slight increase in total phosphorus in the supernatants of the contracted myofibrils as compared with the controls. This was less than 7 per cent and could be accounted for by the presence of the myofibrils in the system and the resulting slight decrease in the volume of the solution to which ATP was added.

The results indicate that the phosphate split from ATP by the contracting myofibril remained in the suspending medium, and therefore large amounts were not permanently bound to the contracted myofibril unless there were an equal movement of phosphate from the myofibril into the suspending medium. The absence of an increase in inorganic phosphorus when ATP was added to the supernatant first removed from myofibrils (line 3 of Table I) indicated that the enzyme or substance responsible for the ATPase action was not in solution, but either intimately associated with or a part of the structure of the myofibril.

Nitrogen content of supernatant.—That the marked structural changes of the myofibril during contraction might be accompanied by a loss of nitrogenous material into the surrounding medium was considered. Previous studies [1] indicated that added ATP could be recovered almost quantitatively as ADP in supernatants of contracted myofibrils. ATP was added to the supernatant removed from the uncontracted myofibrils and the nitrogen determined (line 1 of Table II). This served as a control for the nitrogen determination of the supernatant of myofibrils contracted with ATP (line 2 of Table II).

The results showed no significant change in the nitrogen content of the medium during contraction, confirming the previous studies of recovery of the nucleotide in the supernatant and indicating that there was no significant loss of nitrogenous material into the medium during contraction or, if so,

TABLE II

Nitrogen in supernatants of uncontracted and contracted myofibrils.

	Nitrogen, micrograms per ml		
	Experiment 1	Experiment 2	Experiment 3
1. ATP added to supernatant removed from uncontracted myofibrils	42.3	33.4	51.4
2. Supernatant of myofibrils contracted with ATP.	46.2	35.0	48.8

that it was accompanied by a corresponding shift of nitrogen from the supernatant into the myofibril.

Nitrogen and phosphorus content of myofibrils.—The possibility remained that only a small percentage of the phosphorus split from ATP during contraction combined with or was permanently bound to the myofibril. A second method used to detect this type of change consisted of the determination of total phosphorus and nitrogen content of the myofibrils. Equal quantities of uncontracted myofibrils and myofibrils contracted with ATP were washed five times with 40 mls of KCl-succinate-Mg solution. Half of the material was washed further with ethyl alcohol and ether. All samples were dried, ashed and the nitrogen and phosphorus content determined as described above.

The results of three consecutive experiments are shown in Table III. Each experiment represents the average of several analyses. In samples washed

TABLE III

Total phosphorus and nitrogen of uncontracted and ATP contracted myofibrils.

	Washed with buffer						Washed with alcohol and ether					
	Uncontracted			Contracted			Uncontracted			Contracted		
	Micrograms/mg		P:N	Micrograms/mg		P:N	Micrograms/mg		P:N	Micrograms/mg		P:N
	P	N	Ratio	P	N	Ratio	P	N	Ratio	P	N	Ratio
Exp. 1	1.1	133.8	121.6	1.1	128.7	117.0	0.7	159.2	227.4	0.6	159.0	265.0
Exp. 2	1.0	140.2	140.2	1.0	146.1	146.1	0.7	170.5	243.6	0.7	173.6	248.0
Exp. 3	1.0	150.7	150.7	1.0	151.9	151.9	0.7	164.6	235.1	0.7	162.5	232.1
Average . . .			137.3			138.3			235.4			248.3

with aqueous solution the average phosphorus to nitrogen ratio was 1:137.3 in uncontracted myofibrils and 1:138.3 in ATP-contracted myofibrils. In samples washed with alcohol and ether the ratios were higher, but comparison of the average ratio in uncontracted myofibrils (235.4) with that of contracted myofibrils (248.3) again did not reveal a significant increase in phosphorus content of myofibrils after contraction. These results confirmed those obtained by analysis of the supernatants in which there was no decrease in total phosphorus content.

Adenosinetriphosphatase activity of completely contracted myofibrils

The ability of the isolated myofibril to split off the terminal phosphate group of ATP during contraction was shown previously [15] and in the present study. It became of interest to determine the relationship between this ATPase activity and the actual contractile event. Microscopic observation indicated that addition of a small quantity of ATP to a myofibril suspension resulted in slight contraction. Further contraction could be obtained by addition of more ATP. Addition of an excess of ATP resulted in a round or oval mass without structural cross bands. Subsequent additions of ATP to these fully contracted myofibrils produced no microscopically detectable changes. Following this initial complete contraction, the suspension was centrifuged, the supernatant collected and filtered. The remaining sediment containing fully contracted myofibrils was washed 3 times with KCl-succinate-Mg solution. To this washed sediment containing contracted myofibrils was added the amount of ATP stock solution in KCl-succinate-Mg solution necessary to give a volume and concentration equal to that used to produce the original contraction. The suspension was again centrifuged and the supernatant collected. This entire process was repeated three times. Inorganic phosphorus and total phosphorus were determined in the ATP stock solution, the supernatant of uncontracted myofibrils, and the supernatants removed from the myofibrils, following not only the original addition of ATP but also after each subsequent addition.

The results of two experiments are shown in Table IV. To the fully contracted myofibril ATP was added twice in Exp. 1 and three times in Exp. 2. The total and inorganic phosphorus content of the ATP stock solution was determined (line 2 of Table IV). The initial addition of ATP resulted in contraction, an increase in inorganic phosphorus, and no significant change in total phosphorus. (Compare lines 2 and 3 of Table IV.) Subsequent additions of ATP, although unassociated with structural changes in the myofibril, were accompanied by a similar increase in inorganic phosphorus

TABLE IV

Total phosphorus and inorganic phosphorus in supernatants of completely contracted myofibrils with repeated subsequent additions of ATP.

		Phosphorus, micrograms per ml			
		Experiment 1		Experiment 2	
		Total	Inorganic	Total	Inorganic
1. Supernatant of uncontracted myofibrils		0.9	0.2	0.0	0.0
2. ATP stock solution		52.4	3.7	69.2	3.6
3. Supernatant of myofibrils contracted with ATP		55.1	25.1	67.3	14.3
4. Supernatants after successive ATP additions to contracted myofibrils of line 3	a.	52.9	18.9	63.2	13.3
	b.		19.2	66.4	14.7
	c.			67.3	14.1

(line 4 of Table IV). The results indicated that the fully contracted myofibril retained ATPase activity.

Effect of calcium and magnesium on adenosinetriphosphatase activity and contraction of myofibrils

These experiments were planned to show the effect of magnesium and calcium ions on ATPase in a qualitative manner, since comparative rates of ATPase action under the various conditions were not studied. Only changes in organic and inorganic phosphorus and the presence or absence of contraction were determined. As will be indicated, the effect of magnesium on contraction of myofibrils was more than simply a quantitative one, since in the absence of added magnesium ions ATP did not produce contraction, but in the presence of magnesium ions the addition of ATP resulted in instantaneous complete contraction.

The myofibrils used in this series of experiments were isolated, washed and dialyzed in the cold over a 5 day period. The magnesium content was reduced to about 10 per cent of that of whole muscle. Approximately 2.0 to 5.0 gms of myofibrils were suspended in 20 mls of the KCl-succinate solution in each of a series of tubes. Calcium chloride, magnesium chloride and the sodium salt of ATP were added in different combinations and sequences. The final volume of each tube was then adjusted to 40 mls. The final concentrations of calcium, magnesium and ATP were the same in each tube in which they were added. The ATP was added to give a final concentration

TABLE V

Influence of sequence of addition of ATP, calcium and magnesium upon ATPase activity and contraction of myofibrils.

Sequence of addition					Degree of contraction	Phosphorus in supernatants micrograms per ml			
						Experiment 1		Experiment 2	
Supernatant alone	Myofibril suspension	ATP[1]	Ca[2]	Mg[3]		Total	Inorganic	Total	Inorganic
1						2.8	0.2	0.3	0.2
	1		2		none	2.5	0.3	0.0	0.0
	1			2	none	4.1	0.5	0.0	0.0
	1		3	2	none	4.0	0.3	0.0	0.0
		1				86.5	6.0	63.9	2.5
1		2				86.1	6.9	64.0	3.1
	1	2			none	83.0	20.9	63.4	16.7
	1	2	3		none	83.3	21.1	62.8	17.2
	1	3	2		none	82.1	21.2	62.4	17.3
	1	2		3	none	80.5	34.6	62.9	17.3
	1	3		2	complete	79.6	34.2	62.5	18.4
	1	4	3	2	complete	82.7	35.1	61.4	18.6
	1	4	2	3	complete	82.4	34.6	62.1	18.6
	1	2	4	3	none			63.3	17.5

[1] 850 micrograms/ml.
[2] 75 micrograms/ml.
[3] 83 micrograms/ml. in Exp. 1.
17 micrograms/ml. in Exp. 2.

of 850 micrograms/ml. The final concentration of calcium was 75 micrograms/ml, while that of magnesium was 83.0 micrograms/ml in Exp. 1 and 17 micrograms/ml in Exp. 2. After the appropriate additions a drop of suspension was examined microscopically for the presence or abscence of contraction. The suspensions were then centrifuged. The supernatants were removed, filtered and analyzed for total and inorganic phosphorus. In two instances the supernatants were first removed from the uncontracted myofibrils. The additions were then made to these supernatants and the analyses performed.

The results of two complete experiments of this type are shown in Table V. For purposes of discussion, the experiment can be divided into five major groups as indicated by the single horizontal lines in the table. In the first group the supernatant removed from uncontracted myofibrils served as a

control. Calcium, magnesium and a combination of the two were added to suspensions of myofibrils. The supernatants were analyzed for total and inorganic phosphorus. Under these conditions there was neither contraction of myofibrils nor a significant change in phosphorus content of the supernatant.

The second group consisted of the comparison of the phosphorus content of the ATP stock solution and the supernatant removed from uncontracted myofibrils to which ATP was added. The absence of a significant increase in inorganic phosphorus indicated that the supernatant of the uncontracted myofibrils was free of ATPase activity.

In the third group, the effects of calcium and ATP alone were studied. Addition of only ATP to the suspension of myofibrils did not result in contraction. The increase in inorganic phosphorus indicated that the myofibril retained ATPase action despite the failure to contract. The addition of calcium either before or after the addition of ATP did not result in contraction, and ATPase action persisted.

In group four, the effects of magnesium were determined. The addition of ATP followed by magnesium did not result in contraction, but there was an increase in inorganic phosphorus indicating that the ATP was split before the magnesium was added. This was confirmed by the fact that this supernatant, when added to fresh myofibrils, was incapable of inducing contraction unless more ATP was added. Addition of magnesium followed by ATP resulted in instantaneous, complete contraction. The increase in inorganic phosphorus was identical to that which resulted when magnesium was added after ATP.

The last group consisted of the effects of both calcium and magnesium when added in different sequences. Addition of both calcium and magnesium followed by ATP resulted in contraction and an increase in inorganic phosphorus. The order in which the calcium and magnesium were added did not affect the outcome as long as magnesium was present when ATP was added. Addition of both calcium and magnesium after addition of ATP did not result in contraction.

The results indicated that under the conditions of the experiment myofibrils did not contract on addition of ATP in the absence of adequate magnesium ions. Despite the failure of myofibrils to contract, there was sustained ATPase activity. ATP in the presence of calcium ions did not result in contraction, but again there was ATPase activity. This activity in the absence of calcium or magnesium probably accounted for the failure of contraction to occur when the magnesium was added after the ATP, since presumably

by that time an insufficient amount of ATP remained in the system. At least, the addition to the suspension of more ATP after the magnesium resulted in contraction.

DISCUSSION

The myofibril, after separation from the cell and purification, contracted on the addition of ATP in the presence of magnesium and offered an opportunity to study the chemical reactions associated with this structural and functional change [20, 21]. Studies of the fate of the added ATP revealed that contraction occurred only when the terminal phosphate group was split from the ATP, and that the added ATP was almost quantitatively recoverable in the supernatant as ADP [1]. In the present investigation the fate of the terminal phosphate group split from the ATP was studied. The results failed to indicate any permanent combination of the phosphate group with the contracted myofibril though the conditions of the experiment may have allowed a temporary combination to split before the supernatants could be removed from the contracted myofibrils for analysis. The possibility that the phosphate group split from ATP entered into an exchange reaction with phosphorus already present in the myofibril seems remote, since the ratio of the amount of phosphorus split from ATP to the phosphorus content of uncontracted and contracted myofibrils was approximately 15 to 1. Determination of the nitrogen content of the supernatants indicated that despite the marked change in dimensions and fine structure of the myofibril during contraction there was no uncompensated loss of nitrogenous material into the suspending medium.

Since the only detected chemical change associated with contraction was the result of ATPase action on the substrate, the temporal relationship of this enzymatic reaction to the contractile event was studied. The fully contracted myofibrils retained their ability to split ATP, indicating that ATPase action alone cannot be considered synonymous with contraction. Also, addition of ATP to washed "magnesium-depleted" myofibrils did not result in contraction, even though ATPase action was retained. Thus, the myofibrils split the terminal phosphate group from ATP both before and after contraction. Although contraction was not produced without addition of ATP, ATPase action occurring during contraction was not proved to be an essential part of the mechanism.

Recent studies by Fleckenstein and associates may have a closer bearing on this problem, especially in connection with chemical changes in whole muscle [8]. By analysis of phosphate compounds in intact muscle before

and after electrical stimulation, data were accumulated which might be interpreted to indicate that something in living muscle can either synthesize ATP from ADP at a rapid rate or that there is something more closely related to the process of contraction than the breakdown of ATP.

The demonstration that the highly purified "magnesium-depleted" myofibril contracted only when magnesium was added before or together with the ATP, despite ATPase activity in the absence of magnesium, indicated that magnesium may be essential for contraction and that ATP splitting in the absence of some appropriate ion is not sufficient to initiate contraction. Although ions other than the magnesium may initiate the contractile reaction, calcium and potassium were ineffective under the described conditions.

Under conditions used by Bozler whole strips of muscle extracted with glycerol contracted in the presence of small amounts of magnesium and ATP but large amounts of magnesium depressed ATPase action and produced relaxation [3]. Bozler also concluded that contraction is initiated by bound calcium and that the activity of the contractile elements is governed by the interaction between magnesium and calcium. The relation between the contraction or relaxation of muscle and the action of metals upon the soluble myosin-ATP system has been studied recently by Friess, Morales and Bowen [9]. It is of interest that their myosin has about the same magnesium content as our magnesium-depleted myofibrils. They found that the chelating reagent EDTA seemed to interact with myosin only in the presence of ATP and concluded that the acceleration of myosin ATPase by EDTA is mediated by a metal, possibly magnesium, which is probably firmly fixed by a chemical bond to the protein structure of myosin.

The use in the present study of a suspending medium with pH 6.1 practically excluded the possibility that the ATPase, which is said to be either bound to or a part of the myosin molecule, was the source of the ATPase activity, since myosin ATPase has optimal activity at pH 9.5 and is activated by calcium [22]. Even at pH 7.0, magnesium completely inhibits the ATPase activity of myosin [10]. The combination of myosin-ATPase with actin shifts the pH optimum to 7.7, and under these circumstances magnesium ions produce a five-fold increase in activity [12, 22]. Kielley and Meyerhof [12] have separated from muscle a water-soluble ATPase with a pH optimum of 6.8, which is activated by magnesium. Their enzyme was bound to particulate matter as was true in our experiments. However, there is too little information on the rate of phosphate splitting under different conditions to warrant a definite statement as to which of these enzymes was active in our experiments.

SUMMARY

1. Isolated, structurally intact rabbit skeletal myofibrils contracted instantaneously when ATP was added in the presence of magnesium ions.

2. An ATPase associated with the myofibrils split added ATP, yielding inorganic phosphate and ADP, neither of which was permanently bound to the contracted myofibril. Furthermore, there was no significant loss of nitrogenous material from the myofibrils during contraction unless it was accompanied by an equal shift of nitrogen from the suspending medium into the myofibrils.

3. ATPase was active both before and after contraction. Dialyzed magnesium-depleted myofibrils did not contract but split added ATP, indicating that ATP-splitting alone is not a sufficient event to cause contraction. Fully contracted myofibrils retained ATPase activity despite the absence of structural changes accompanying this activity.

4. Dialyzed magnesium-depleted myofibrils contracted instantaneously and split ATP when first magnesium ions and then ATP were added; but when calcium ions and then ATP were added, contraction did not occur despite ATPase activity.

5. There was no evidence of antagonism between these ions, since myofibrils to which were added both calcium and magnesium ions followed by ATP contracted and split ATP.

6. Although contraction was not obtained without the addition of ATP, it has not been shown that ATP splitting is essential for contraction.

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THE MECHANISM OF NICOTINE-INDUCED POLYSPERMY

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Within a second or two after the attachment of the fertilizing sperm to the surface of a sea urchin egg at room temperature, the cortical reaction, a series of visible surface changes begins to sweep over the egg surface [11, 12, 15, 19]. These visible changes are preceded and initiated by the self-propagating fertilization impulse, a submicroscopic change of similar rate which spreads over the egg surface as a chain reaction from the point of sperm attachment [2, 3, 13, 19]. The cortical reaction takes about 20 seconds to cover the egg surface [15].

During and immediately after the maturation divisions, sea urchin eggs gradually develop a block to polyspermy, the mechanism of which has previously been discussed as possibly related to some component or components of the cortical reaction [2, 4, 8, 9, 12, 13, 15]. The results of earlier experiments by Rothschild and Swann [15, 16, 17] were not incompatible with the view that the cortical reaction might be identical with the block to polyspermy, provided the proportion of successful hits of the spermatozoon on the egg surface was considerably less than one. In later work, Rothschild and Swann determined that this proportion was actually less than one, but not sufficiently small to account for a block to polyspermy as slow as the cortical reaction [17]. Accordingly, a faster block was sought, and some evidence for this found in the sudden decrease of the fertilization parameter or α value [18], during the propagation of the block to polyspermy. However, the α value is only a measure of the success of sperm-egg contacts without determining the cause for their success or failure. Thus the drop in α value might be due to a number of changing factors, some of which were already mentioned [16, 18]. A subsequent paper by Rothschild on the induction of polyspermy by nicotine was also an attempt to demonstrate the existence of a block to polyspermy which would be more rapid than the cortical reaction [14]. As will be stated below the present writers disagree

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in a number of points with the conclusions reached by Rothschild and Swann, for without proving that sperm retain constant fertility and that the egg surface has a homogeneous threshold, α values cannot be interpreted as indicative of a rapid propagated block to polyspermy.

Rothschild and Swann [18] introduced a method of determining the rate of fertilization, see also [10]. An improved technique by one of the writers [7, 4] involves the interruption of the fertilization process at any time after insemination by the addition of a spermicidal detergent in such low concentrations that the eggs are not apparently affected. By interrupting fertilization of potentially polyspermic eggs at different times after insemination, it is possible to examine eggs which have been entered by only one sperm, but which at the same time are susceptible to further penetration by a sperm which subsequently attaches itself to the egg surface.

MATERIAL AND METHODS

Mature gametes of the sea urchin, *Psammechinus miliaris*, from Kristineberg on the Swedish west coast were obtained in the usual manner [1, 4]. Eggs both with and without their jelly layers were used. Acid treatment (pH ~5 for about 30 seconds) was used to deprive the eggs of their jelly layers.

Eggs were pretreated with nicotine (Eastman Kodak Company, lot no. 1242) in concentrations from 1/1000 to 1/75,000 (volume/volume) for periods from one to ten minutes. In some experiments, fertilization was interrupted with 0.0007 per cent sodium laurylsulfate, a spermicidal detergent. Experiments were arranged in such a way that the nicotine concentration was reduced 100-fold when the detergent was added. When the procedure was followed by washing after the eggs had settled, the effects of nicotine on cleavage, and any possible combined effects of nicotine and sodium laurylsulfate were minimized.

Eggs were considered polyspermic only if superfluous sperm could be seen in the cytoplasm *before the first mitotic division occurred.* The recognition of supernumerary sperm is not always easy; extra nuclei are best seen with a water-immersion lens (22 or 50 ×). Counting polyspermic eggs on low power photomicrographs cannot yield accurate data because abnormal divisions closely resembling polyspermic cleavage take place in nicotine-treated, monospermic eggs.

RESULTS

The influence of nicotine on the sperm-egg interaction.—Jelly-free eggs pretreated 2 minutes with 1/2000 to 1/8000 nicotine collected vast numbers of sperm at their surfaces within seconds after insemination, in contrast to the untreated jelly-free eggs which did not collect supernumerous sperm (Fig. 1 a and b). When the jelly was left intact, this difference between nicotine-

treated and untreated eggs was not so striking, for the jelly itself tended to trap a large number of sperm which would otherwise have reached the egg surface. There was no indication that nicotine caused the eggs to become more adhesive to one another or to glass surfaces. Eggs treated with nicotine tended to lose their jelly layers more readily than the controls.

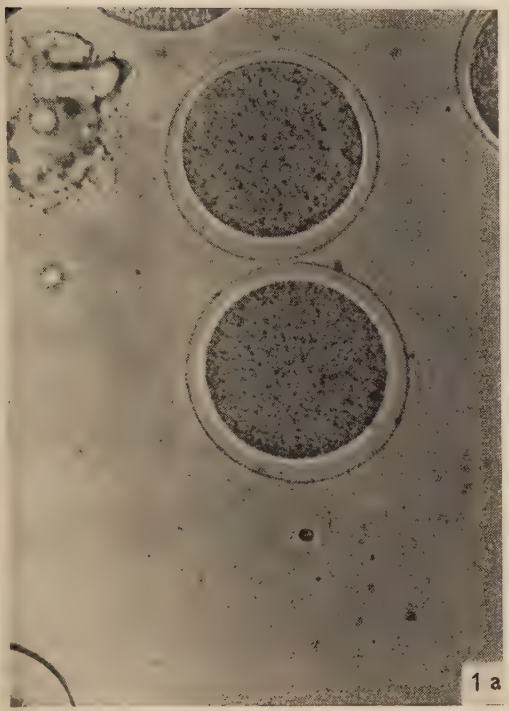
The influence of nicotine on membrane elevation and the thickness of the hyaline layer.—Eggs exposed for 10 minutes to concentrations of nicotine from 1/1000 to 1/8000 were examined for the height of their elevated fertilization membranes. Eggs fertilized following pretreatment with higher concentrations of nicotine had a very low thin membrane; those pretreated for shorter times or with lower concentrations were more nearly like the controls (Fig. 1, cp. *a* and *b*).

The hyaline layers of eggs pretreated with nicotine did not increase in thickness so markedly as in the control eggs. Several minutes after fertilization, the hyaline layer thickness could be correlated with the concentration of nicotine used in pretreatment. In some experiments 5-10 per cent polyspermy was obtained after 10 minutes pretreatment with 1/20,000 v/v nicotine using 3.5×10^6 sperm/ml. At this concentration there was no visible damage to the hyaline layer.

The effect of nicotine on the cortical reaction.—In the experiments to be described, a procedure was followed for obtaining eggs which were monospermic, yet potentially susceptible to polyspermy. In this way, it proved possible to determine what state of the cell surface would permit superfluous spermatozoa to penetrate. Jelly-free eggs were pretreated 2 minutes with 1/1000 to 1/8000 nicotine and inseminated. At intervals of 5 or 10 seconds, aliquots of mixed gametes were transferred to an excess of sea water containing the detergent, sodium laurylsulfate (0.0007 per cent). In the early-transferred aliquots, a portion of the eggs were monospermic, and the rest were unfertilized. In the aliquots stopped some seconds later, there were eggs in various states of partial fertilization.[1] As the time interval between insemination and interruption of fertilization increased, the number of partially fertilized eggs increased, then decreased, and first totally fertilized, then polyspermic eggs became dominant.

Many eggs from the 5, 10 and 15 second aliquots showed small areas of limited cortical granule breakdown when viewed with a 50 × water immersion objective; beneath these restricted areas, sperm nuclei were observed. These sperm nuclei came to rest just beneath the cortex, and no sign of aster formation or migration was detected. Often more extensive cortical granule

[1] Partial fertilization results from interruption of the cortical reaction. See also [1, 4, 5].



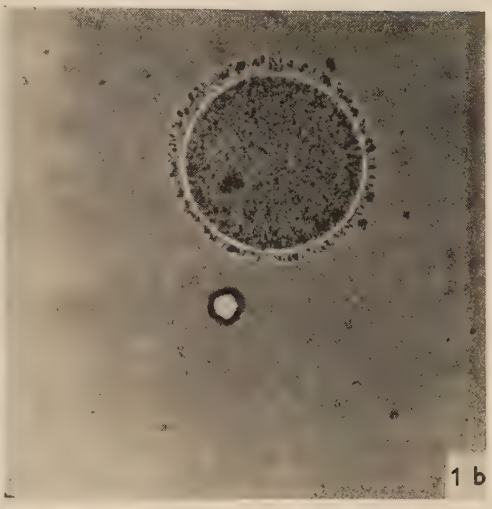


Fig. 1. Photographs showing the difference in sperm-egg adhesiveness between (*a*) control, untreated eggs, and (*b*) nicotine-pretreated eggs inseminated with the same sperm density at the same time.

Fig. 2. A photograph showing a "membrane-blister" over an area on the surface of a nicotine-pretreated egg. The cortical granules were intact on all parts of the surface except beneath the membrane.

breakdown took place, especially at lower nicotine concentrations. As a consequence of incipient membrane elevation many blister-like membranes were seen covering areas of restricted cortical granule breakdown (Fig. 2). The centers of these blisters were often seen to have been pierced by a sperm tail. In such cases the cortical change had been interrupted as a consequence of the pretreatment. It was noted, however, that occasionally there would be a secondary cortical granule breakdown in partially fertilized eggs from 10 to 30 minutes after the first cortical reaction had been interrupted. This secondary breakdown was not a resumption of the cortical reaction, for single granules broke down in irregular fashion over the course of several minutes at random points on the surface; the presence or absence of nicotine seemingly had little to do with the process (cf. [4]).

Some partially fertilized eggs were observed which contained more than two sperm nuclei. In some cases, particularly at high nicotine concentrations, (1/1000–1/2000 v/v for 2–5 minutes) eggs were seen with several islands of cortical granule breakdown scattered over the surface. Some eggs were seen which had 40–50 per cent of the surface covered by a cortical reaction evidently initiated by one sperm; on the side not affected by this partial cortical reaction there were several islands of restricted cortical granule breakdown, each containing one sperm head buried just beneath the cortex.

Viewed under dark-field illumination, the surface of eggs with islands of restricted cortical granule breakdown exhibited patches of thin, silvery-white or faded-yellow against the background of the thick, yellow-orange unfertilized surface.

The fertilization and polyspermy rates of nicotine-pretreated and control eggs.—In a study of polyspermy, it is essential to know when the second sperm enters. Interruption of fertilization by treatment of aliquots of fertilizing gametes with sodium laurylsulfate provided data on the rate of fertilization and polyspermy such as those plotted in Fig. 3. These data are typical of those obtained in several experiments of this type except that different lots of eggs differed somewhat in their susceptibility to the polyspermy-inducing action of nicotine.

It is clear from Fig. 3 that eggs became polyspermic up to and considerably after the normal time for completion of the cortical reaction. Although much of the early polyspermy could be attributed to failure of the cortical reaction to cover the surface completely, examination of the eggs pretreated with nicotine and stopped with detergent at 40 seconds in this experiment showed almost 100 per cent membrane elevation, indicating that the cortical reactions in nearly all of the eggs were then complete. Yet, at 40 seconds, the

percentage of polyspermy was scarcely more than half the value it later attained.

An experiment was designed to determine whether nicotine-pretreated eggs could be penetrated by supernumerary sperm even considerably after the cortical reaction had covered the surface. Eggs were pretreated for two minutes with 1/2000 nicotine and inseminated lightly (4×10^5 sperm/ml);

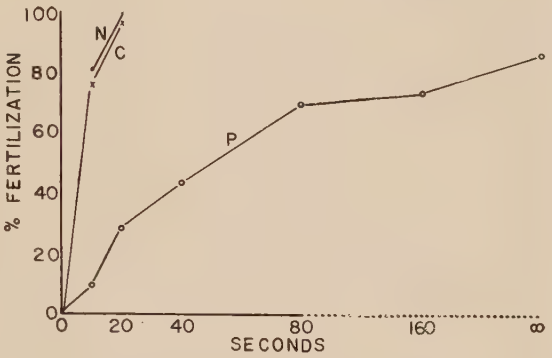


Fig. 3. The rate at which control eggs (*C*) and nicotine-pretreated eggs (1/2000 for 2 minutes) (*N*) became fertilized when inseminated at sperm density 7×10^6 sperm/ml. (*P*) represents the corresponding rate at which the nicotine-pretreated eggs became polyspermic; the controls exhibited no polyspermy.

after two minutes, an aliquot of the fertilizing gametes stopped with detergent showed that at that time 98 per cent of these eggs had been fertilized; of these, 5.5 per cent were polyspermic, and less than 5 per cent were partially fertilized. At 3, 4 and 5 minutes respectively were 26.3, 28.2 and 32.0 per cent of the eggs polyspermic (300 eggs counted in each series). Control eggs treated in the same manner except for nicotine-pretreatment failed to exhibit any polyspermy. Unfertilized eggs inseminated before and after the experiment with the same heavy sperm suspension (3.5×10^7 sperm/ml) showed 2 per cent polyspermy. This experiment confirmed the inferences drawn from the polyspermy rate data that nicotine at the concentration used interferes with the establishment of the slow, complete block to polyspermy.[1] This result is in contradiction to statements by Rothschild [14].

The influence of nicotine on cleavage and the recognition of polyspermy.— The first experiments in this series on polyspermy were performed using either the presence of supernumerary nuclei or abnormal cleavage (when the untreated controls were normal) as criteria of polyspermy. It was soon realized that abnormal cleavage could not be used as a criterion of polyspermy in experiments involving nicotine. (This may be true for other poly-

[1] It is clear that the sperm must enter nicotine-pretreated fertilized eggs through their weak fertilization membranes. The formation of the membrane is delayed and impaired after treatment with nicotine (cf. Fig. 1 *b*).

spermy-inducing substances as well.) Nicotine often suppressed the first cleavage of monospermic eggs, causing at first the appearance of eggs with supernumerary nuclei and then abnormal cleavage, for eggs in which the first nuclear division had taken place but in which the cytoplasmic cleavage had failed often cleaved directly into four blastomeres. The appearance of such four-cell stages was easily confused with that of some first cleavages of polyspermic eggs.

After much trial and error, it was decided that only the presence of an extra sperm nucleus before mitosis could be an accurate criterion of polyspermy. Eggs were fixed in 4 per cent formalin at the time when the normal eggs were forming spindles. Fixation caused the nuclei to swell slightly, and allowed more time for the examination of each egg counted. Thus possible confusion between abnormal cleavage caused by polyspermy and by nicotine was avoided (cf. [14]). Fixation was not necessary if only a few hundred eggs needed to be counted before the first cleavage.

DISCUSSION

According to Rothschild [14], nicotine-induced polyspermy could be attributed to two separate actions of this drug: (1) an effect causing a 3.5-fold increase in the chances for the success of any sperm-egg collision, and (2) specific inhibition of a rapid (2–3 second) block to polyspermy.

The definite tendency for sperm to adhere irreversibly to the surface of nicotine-pretreated eggs might provide at least a partial explanation for the increased success of sperm-egg contacts. It is probable that the 3.5-fold increase in this tendency measured by Rothschild was too conservative for it seems clear that he did not count partially fertilized eggs (eggs with "membrane blisters") in his data, and it is likely that many of his "unfertilized" eggs actually contained sperm nuclei which had not initiated a cortical reaction.

Although the effect of nicotine on membrane elevation is worthy of note, it is not clear whether this effect has anything to do with the block to polyspermy. The fertilization membrane is not generally credited with an important role in preventing polyspermy [15], but the failure of the membrane to elevate properly is probably an indication of abnormalities in the cortical reaction itself.

The ease with which sperm could enter the unfertilized cortex of partially fertilized eggs (not only those obtained by nicotine treatment, but also by heat and stretching [4, 2]) indicated the importance of this failure of the

cortical reaction in evaluating the mechanism of the block to polyspermy. It is clear that most nicotine-induced polyspermy can be attributed to outright failure of the cortical reaction. This conclusion is in contradiction to the statement by Rothschild that the cortical reaction is unaffected by nicotine. However, the papers of Rothschild and Swann [16] and of Rothschild [14] on nicotine contain respectively (1) a series of darkfield photographs showing the entrance of a second sperm into an egg which showed clear inhibition of the cortical reaction initiated by the first sperm (cf. [16], p. 405), and (2) a description of some obviously partially fertilized eggs obtained by plunging nicotine-pretreated eggs into hypotonic sea water just after sperm entrance ([14], p. 62).

The ability of sperm to enter nicotine-pretreated eggs as late as 3, 4 or 5 minutes after the entrance of the first sperm, at a time when the "slow complete block" should have been completed, seems to be related to the extremely poor hyaline layer development observed after nicotine-pretreatment. The role of the hyaline layer in the establishment of a final block to polyspermy has previously been indicated, since removal of the hyaline layer by enzymatic digestion can permit refertilization [9]. The role of the hyaline layer in the prevention of polyspermy has been referred to elsewhere [4, 8]. It is noteworthy that attempts to bring about refertilization by post-fertilization nicotine-treatment failed. One might conclude that nicotine more likely inhibits some step in the formation of the hyaline layer than dissolves or attacks the layer already formed.

The problem of how most eggs successfully resist polyspermy when bombarded by extremely heavy concentrations of sperm has by no means yet been solved. The defense mechanism against polyspermy probably does not reside wholly within the egg. The increasing incidence of sperm-sperm interactions reducing the effective sperm concentration at high sperm densities has been pointed out by Rothschild [17]. It is known that eggs release acid upon fertilization; one of the effects of acid might be the reduction of sperm motility and fertility. Local threshold differences at the egg surface [5] might exhibit themselves as different susceptibilities to sperm, or as different latent periods at various parts of the egg surface. Sperm entrance at a point of low threshold might give the egg the necessary seconds to complete its cortical reaction if the rest of the egg surface required a longer latent period. Although the importance of the angle at which the spermatozoon hits the egg surface has been discussed before [20], this matter has become conspicuous in the light of the recent work of Dan [6], who showed that an acrosomal thread may participate in sperm penetration. Conceivably, discharge of this thread

prematurely or in the wrong direction would remove the effectiveness of a particular sperm. Thus steric factors and the short duration of some sperm-egg contacts may be reasons for their lack of success.

From the available evidence, it seems plausible to ascribe the major polyspermy-preventing role to the cortical reaction and the growth of the hyaline layer which follows immediately. The relative importance of sperm-sperm interactions, sperm paralyzing substances, the angle at which the spermatozoon hits the egg surface, contact time and other similar matters can only be the subject for further experimentation. At this time, there seems to be no definite indication of the presence of a block to polyspermy on the part of the egg which is faster than the cortical reaction; in fact, evidence so far tends to speak against any such rapid block.

SUMMARY

The effect of nicotine on sea urchin eggs with and without jelly coats was examined.

Sperm adhere irreversibly to the surface of nicotine-pretreated eggs, thus perhaps partly accounting for the increased probability of a successful sperm-egg interaction.

The height of membrane elevation was considerably reduced by nicotine-pretreatment. This effect is probably of more importance as a symptom of the injurious effect of nicotine on the egg cortex than as indicating any direct influence on the block to polyspermy on the part of the membrane.

Nicotine-pretreatment of sea urchin eggs often predetermines the failure of the cortical reaction on subsequent fertilization. Many eggs containing one or more sperm nuclei were found to be susceptible to polyspermy, largely by virtue of the partially fertilized character of their surfaces.

Nicotine-pretreated eggs admit supernumerary sperm long after the block to polyspermy would have been complete in the controls. This was correlated with the facts that (1) the hyaline layer development was extremely poor even in completely fertilized nicotine-pretreated eggs, and (2) the hyaline layer has been suggested to play an important role in the establishment of the block to polyspermy.

We are deeply indebted to Professor John Runnström for his interest and guidance in this work, and to Dr. Gunnar Gustafson, the Director of the Kristineberg Zoological Station, for providing us with excellent working conditions and material. We should also like to thank Lord Rothschild and Prof. Clement Markert for criticizing the manuscript.

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STUDIES ON THE FERTILIZATION OF JELLY-FREE SEA URCHIN EGGS[1]

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The role of the jelly coat in the fertilization of the sea urchin egg has been intensively discussed since Lillie [10] presented his "fertilizin-theory". Lillie [10] assumed that the gelatinous layer covering the sea urchin egg contained a substance, "fertilizin", which is essential for fertilization. He observed that sea water that had been in contact with sea urchin eggs contained fertilizin and that this "egg-water" agglutinated the sperm of the homologous species. Furthermore he found that sperm motility was increased upon the addition of egg-water. Lillie assumed that the jelly coat was saturated with fertilizin, which substance is secreted by the egg. However, it was observed that the jelly slowly dissolves when the eggs are stored in sea water, and it was also found that a jelly coat solution obtained by treating the eggs with acidified sea water exhibits the same properties as does the egg-water (cf. e.g. [15]).

Hartmann and Schartau [8] introduced the term "gamone" for the different factors secreted by the gametes. The literature in this field is very extensive and the reader is referred to the excellent study by Vasseur [17], which gives an account of this literature until 1952. Most of our knowledge about the chemistry and physiology of the jelly coat we owe to Vasseur's work [15, 16, 17, 18, 19].

However, there is still some confusion about the action of the jelly coat in fertilization. It has been claimed that the jelly is almost indispensable for fertilization and that the removal of the jelly causes a decrease in fertilizability [14]. To bring about fertilization of jelly-free eggs it is necessary to use more sperm than in the corresponding control [14]. It seems rather doubtful to the present author if this difference is depending upon the lack of jelly, since the treatment with acid sea water (pH 3.5) might cause injury to the egg even if the time of exposure to the acid medium is very short.

It was shown by Runnström and Wicklund [12] that membrane formation is impaired by addition of jelly solution. Vasseur and Wicklund found that the membrane inhibiting action of jelly solution is heat labile, probably depending upon depolymerisation of the substance [19]. Previously Runn-

[1] Presented before the Symposium on the Physiology of Fertilization, Palermo, April 26–30, 1955.

ström *et al.* [13] showed that very compact jelly coats present a certain obstacle to fertilization and it was also demonstrated that the removal of the jelly coat made the eggs fertilizable. All these facts indicate that the jelly, besides its activating influence on the sperm [1, 18, 19], also exerts an impairing action on fertilization.

As it could be suspected that the mode of removal of the jelly coat might influence the fertilizability of the egg the present study was made in order to find a method for dissolving the jelly without causing injury to the egg. The influence on the egg and on fertilization was determined according to the fertilization rate method previously described [2].

EXPERIMENTAL

Several sea urchin species were employed in this work which was carried out in Naples at the Stazione Zoologica and at Kristinebergs Zoological Station at the Swedish west coast. In Naples *Paracentrotus lividus* and *Arbacia lixula* served as material and at Kristineberg *Psammechinus miliaris* and *Echinocardium cordatum* were used. The experiments were carried out during February-August 1954.

The gametes were obtained in the usual manner [2] and the concentration of sperm was 1.5×10^6/ml in all experiments. To obtain a material as homogenous as possible eggs from only one female were used in each experiment.

To free the eggs from their jelly coats an appropriate amount of 0.1*M* hydrochloric acid was added to a beaker containing 50 ml of sea water and then 100 ml of egg suspension was poured into the beaker containing the acid sea water. Thus the eggs were never exposed to the undiluted acid and the mixing of eggs and acid was very rapid.

Vasseur [15] previously investigated the appropriate pH for jelly removal in many sea urchin species, and he found that a pH of 5.5–5.8 was sufficient to remove the jelly in most species. It was also demonstrated that in the pH interval 4.0–5.0 the jelly dissolved but simultaneously some of the eggs became cytolysed. Many workers have used a still lower pH to remove the jelly coat and it must be assumed that the eggs will be affected rather severely at this pH (3.5) although the exposure to this high concentration of acid has been of a short duration.

Several experiments were made to determine the optimal concentration of acid. In *Paracentrotus* and *Psammechinus* a pH of 5.8 was found to give a complete removal of the jelly coat. In *Arbacia* a sligtly higher pH, 5.9–6.1, was satisfactory and in *Echinocardium* the jellies were removed at pH 5.5. The removal of the jelly was performed in wide beakers and the eggs had only to sediment 20 30 mm. After 30–180 seconds the supernatant was rapidly sucked off by a water pump and a large quantity of filtered sea water was added.

In order to elucidate the action on the egg of different concentrations of HCl several preparations of jelly solution were made from the same egg material. The eggs as well as the jelly solutions were collected and tested according to the fertilization rate method [2].

It is essential to get the eggs absolutely freed from the jelly coat; no traces of jelly adhering to the egg surface were tolerated. Before each experiment a very dense egg suspension was examined in the phase microscope with the oil immersion objective. The eggs were lying close together and no space was seen between adjacent eggs. Furthermore, part of the jelly free eggs was stored in a small amount of sea water in order to produce "egg water". After one hour the egg water was tested for its sperm agglutinating capacity. In none of the experiments in which the eggs had been subjected to a pH of 5.5–6.1 was any sperm agglutination observed. This lack of agglutinin also indicates that the eggs were jelly free [9].

RESULTS

The removal of the jelly coat at a pH of about 5.8 had a very striking effect on the fertilization rate. The fertilization of the intact control eggs was much slower than that of the jelly-free eggs (cf. Fig. 1). Membrane formation, cleavage and development was quite normal in the jelly-free eggs. As a matter of fact membrane formation in *Arbacia* eggs often was improved by removing the jellies (cf. [13]).

When the eggs had been treated at a lower pH, e.g., 4.5–5.0, the fertilization rate was impaired as compared with the control. In material treated at pH 3.5–4.0 only a minor part of the eggs (i.e., 20–40 per cent) was fertilized. The membranes were low and granular and cleavage abnormal. These eggs were evidently affected by too high a concentration of HCl.

Some preliminary experiments were also made with the jelly solutions obtained. Before the experiments the pH of the jelly solution was adjusted

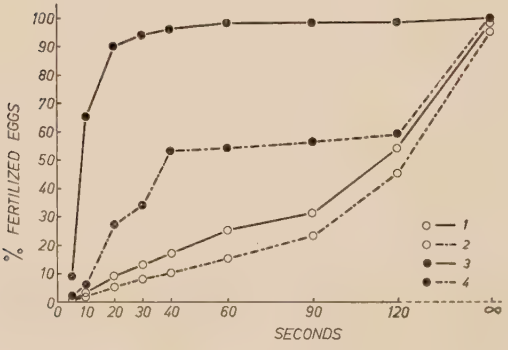

Fig. 1. Eggs and sperm from *Paracentrotus lividus*.

1. Control. Untreated eggs with jelly coats. *2*. Eggs with jelly coats. To 150 ml of egg suspension, 4 ml of a viscous jelly solution were added immediately before insemination. *3*. Jelly-free eggs. *4*. Jelly-free eggs. To 150 ml of egg suspension, 4 ml of a viscous jelly solution were added immediately before insemination.

In *3* and *4* the eggs were freed from their jelly coats by treatment with HCl for 180 seconds at a pH of 5.8.

The jelly solution used in *2* and *4* was obtained by lowering the pH of a dense egg suspension to 5.8. (eggs from *Paracentrotus lividus*). The supernatant (=the jelly solution) was centrifuged for 30 minutes at 3500 × g and the pH adjusted to 8.0 before the experiment.

5, 10, 20 ... 120 seconds after insemination sodium-lauryl-sulphate was added to a final concentration of 0.001 per cent.

Each circle of the curves represents 250–300 counted eggs.

to 8.0 by adding 0.1*M* NaOH. The jelly solutions used in these experiments contained about 0.06–0.08 per cent high-molecular substance.

It was found that a jelly solution prepared at pH 4.5 enhanced the fertilization rate of both the control and the jelly-free eggs (the jellies of these eggs were removed at pH 5.8). On the contrary, jelly solutions prepared at pH 5.8 impaired the fertilization rate (cf. Fig. 1). A tentative interpretation of these experiments is that a high but not a low concentration of acid effects a dissolution of substances from the egg. As was already shown, too low a pH causes cytolysis [15] and the presence of proteins in the jelly solution might improve fertilization (cf. [2]). In connection with a disintegration of the egg a release of antifertilizin (JePptF(E)) might also occur. JePptF(E) has been found to improve the fertilization rate and to counteract the sperm agglutinating capacity of jelly solution (cf. [5]). In the case of jelly solutions prepared at pH 5.8 no cytolysis was observed and the high final fertilizability of the eggs after such treatment also indicates that no injury to the eggs was produced. However, more work is needed to elucidate the influence of the mode of preparation on the physiological activity of jelly solution. The experiments referred to above were all carried out at rather high concentrations of jelly coat solution. Therefore experiments must also be made at lower concentrations as it can be suspected that at these concentrations the stimulating effect on sperm motility might predominate.

CONCLUSIONS

Though our knowledge about the physiological properties of the jelly coat (=fertilizin) is still incomplete it must be concluded that the fertilizin-theory [10] is no longer tenable. The jelly coat is not only dispensable to fertilization but it acts in fact as a barrier to fertilization.

Since Runnström *et al.* showed [11, 12] that jelly solution inhibits membrane formation, it became difficult to regard the jelly as fertilizin, i.e. the substance promoting fertilization. From experiments with substances chemically related to the jelly coat it appears that they all inhibit fertilization when applied in sufficient concentrations [6, 12, 20]. Like the jelly coat these substances exert an activating influence on sperm motility.

The jelly coat protects the egg against mechanical injury and aids the egg in maintaining its chemical milieu. The species-specificity in sperm agglutination is very evident [16], and it has also been demonstrated that the jelly coat acts as a block to cross-fertilization [7]. The agglutination itself may

cause a decrease in the actual concentration of sperm which may serve as an additional block to supernumerary spermatozoa (cf. [3, 4]).

It has also been demonstrated that all the substances used in order to improve fertilization act swelling and dissolving on the jelly coat (cf. [2]). Under certain conditions a precipitation of the strongly acid jelly coat also improves fertilization [5]. These facts indicate the negative influence exerted on fertilization by the jelly coat *in situ*.

SUMMARY

Removal of the jelly coat from the sea urchin egg at pH 5.8 improves the fertilization rate.

Addition to the egg suspension of the jelly solution obtained at pH 5. 8 causes a decrease in the fertilization rate.

Removal of the jelly coat at a lower pH results in an injury of the egg.

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CHANGES DURING INTERPHASE IN NUCLEIC ACID AND PROTEIN CONTENT OF *TRADESCANTIA* ROOT TIP NUCLEI

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At anaphase of cell division, when the chromosomes separate, the total content of the nuclear unit is halved, and it increases again at some time prior to the next division. Of the complex chemical components of the nuclei desoxyribonucleic acid (DNA) has been the subject of the most intensive study, partially because it is felt to be part of or associated with the gene itself, and partially because the available techniques are best adapted to DNA measurements. Studies of the DNA content of interphase nuclei have led to the conclusion that it tends to be constant [1, 16, 19, 22, 23], excluding certain exceptions [2, 13, 17, 20], and therefore doubles exactly between cell divisions. The doubling is felt to take place, depending upon the material studied and/or the technique used, at a fairly uniform rate through interphase [8, 22, 24] or slowly at first and then very rapidly just before prophase [17, 18] or immediately after telophase [10, 18].

Analyses of the total content of interphase nuclei using the ultraviolet absorption technique have been restricted to nucleic acid studies [11, 12, 24] because it was found or assumed that nucleic acid absorption was so high that protein absorption could not be measured.

Preliminary study of ultraviolet absorption spectra of root tip nuclei of *Tradescantia paludosa* showed that some contain a very high proportion of proteins so that protein absorption sometimes even exceeds nucleic acid absorption. Root tip nuclei of this plant are, moreover, large (nuclear diameters frequently exceed 20μ) and relatively homogeneous. The present study of these nuclei was undertaken with two objectives: (1) to follow the change in nucleic acid and protein content of nuclei through the period of interphase growth, and (2) to compare nuclei from actively dividing cells of the meristematic tip with those from non-dividing cells of the differentiated area.

MATERIALS AND METHODS

The nuclei studied were isolated from root tips of *Tradescantia paludosa* (E. Anders and R. E. Woodson), using Dr. A. Orville Dahl's clone 6-50. The tip 2.5 mm of

roots ("mitotic area") contains many dividing cells as well as differentiating cells that are no longer in the mitotic cycle, while the second 2.5 mm ("differentiated area") contains only differentiating or differentiated non-dividing cells. Root tips were cut into two pieces 2.5 mm in length and frozen and dried using the apparatus and technique described by Moberger *et al.* [15]. After drying the root pieces were placed separately into dry glycerine for a period of six hours during which time the glycerine seeped into the tissue. They were then macerated with a spear-tipped needle, and a drop of the resulting macerate placed on a quartz slide. The material on the slide contianed whole cells, cell walls, cytoplasmic debris, and nuclei, most of which were free of cytoplasmic tabs. A few representative nuclei of the two root tip regions appear in Fig. 1.

Nucleic acid and protein content of the nuclei was measured using the ultraviolet microspectrophotometric technique as outlined by Caspersson [3, 5]. The instrument employed was the model described by Caspersson in 1950 [6]. The method was, in brief, as follows: Each nucleus was scanned across its widest diameter by separate beams of several wave lengths of ultraviolet light 2μ wide. The wave lengths used were 2650, 2750, 2800, 2894, and 3150 Å. The extinction of the nucleus at each wave length was determined by averaging the extinctions of 11 equally spaced points within the center 50 per cent of the nuclear path. Each nucleus was also photographed at a known magnification and its area measured with a planimeter.

The amount of non-specific light loss was determined from the measurements at 3150 Å, a wave length at which it is assumed that all loss is non-specific. The 3150 Å extinction was subtracted from shorter wave length extinctions to correct them for non-specific loss. Measurements were restricted to nuclei showing a low 3150 Å extinction in order to minimize as much as possible the difference between the correction used and that that should have applied if Rayleigh scattering were taking place. The method of preparation resulted in nuclei whose 3150 Å extinction was usually below 0.1, and only 7 of the 86 used showed an extinction at this wave length slightly in excess of 0.1.

The absorption spectrum for each nucleus determined from the scannings was analyzed by a method of curve fitting in order to evaluate the quantity of nucleic acids and protein present. The procedure used actually does not show how much nucleic acid and protein was present, but does show a combination of desoxyribonucleic acid plus the amino acids tryptophane and tyrosine that would give the same absorption spectrum as was obtained from the nuclei. To do this the protein in the nuclei was assumed to contain 1.7 parts tryptophane to 2.6 parts tyrosine, a figure shown by a survey of amino acid contents to be typical of bulk proteins of plants of a number of different families [14]. The extinction of tyrosine is influenced by its degree of dissociation (4, 9). A 25 per cent dissociated tyrosine molecule was found by trial and error to be the most suitable for use in these calculations, for the observed nuclear extinctions could not be fitted when the extinction curves for more or less highly dissociated tyrosine were used. The extinctions to be expected from a solution of 1.7 parts tryptophane to 2.6 parts 25 per cent dissociated tyrosine at a total concentration of 10^{-10} mg per μ^2 as derived from Holiday [9] is recorded in the first column of Table I. It is likely that some ultraviolet light is also absorbed by other amino acids in the nulcei, but since they absorb strongly only at wave lengths shorter than those used for these measurements, their absorption could not be evaluated,

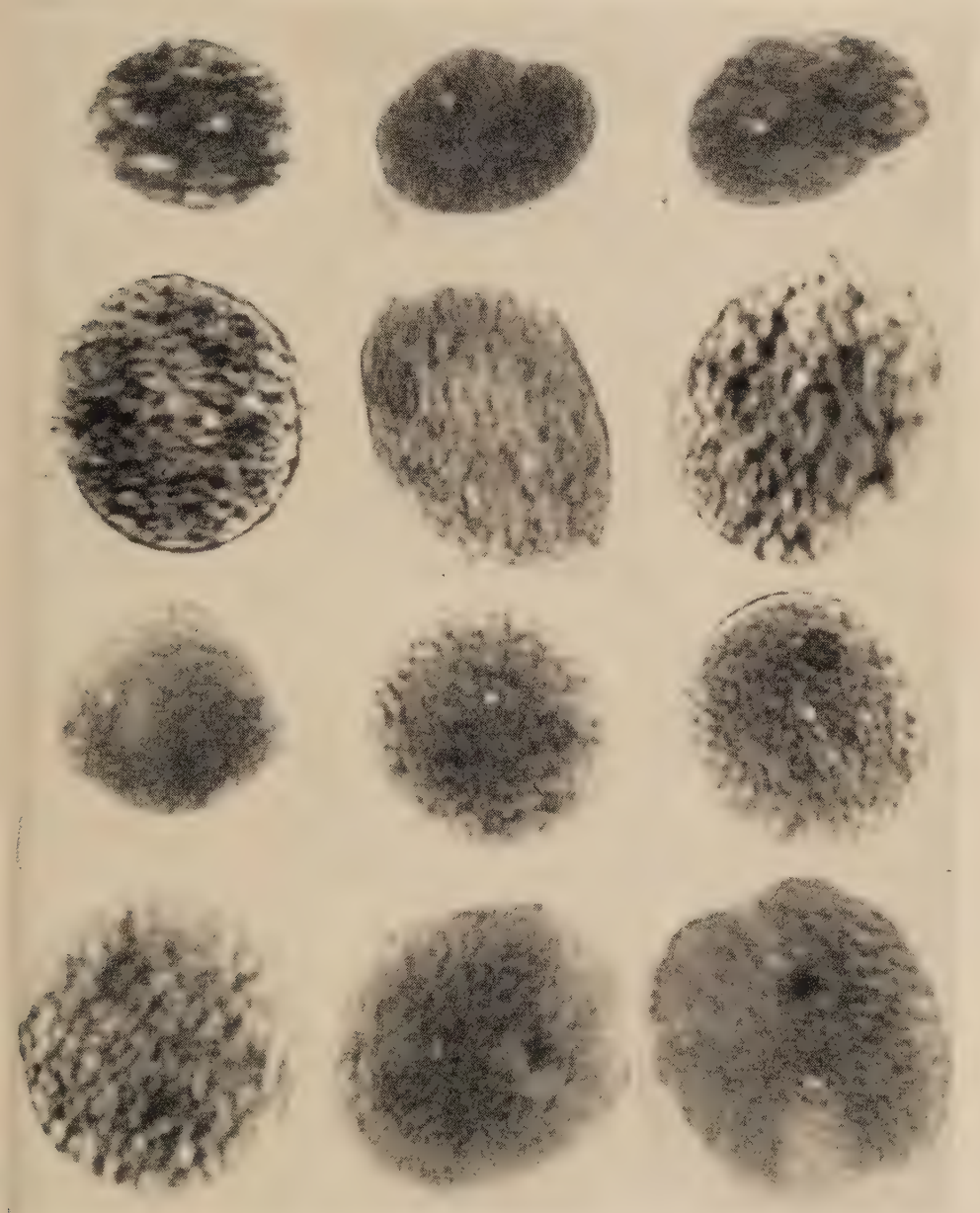


Fig. 1. The appearance of typical isolated nuclei photographed with ultraviolet light at 2650 Å. The six upper nuclei were isolated from the mitotic area and the lower ones from the differentiated area. Magnification is ca. 2300×.

Table I

Typical examples of fit between nuclear extinction coefficients and combined extinctions of solutions of nucleic acid, tryptophane, and tyrosine.

Wave length	1.7 trypto-phane: 2.6 25 per cent dissoc. tyrosine*	DNA*	Nucleus from tip 2.5 mm		Nucleus from second 2.5 mm	
			Observed**	Calculated	Observed**	Calculated
2650	0.123	0.210	0.504	0.504	0.290	0.290
2750	0.141	0.150	0.504	0.501	0.276	0.275
2800	0.143	0.120	0.481	0.481	0.259	0.259
2894	0.090	0.060	0.269	0.290	0.147	0.153
3150	0.003	0.000	0.000	0.008	0.000	0.003

* $\times 10^{-10}$mg per μ^2.
** After subtracting non-specific light loss.

and so was not considered in the calculations. The second column of Table I shows the extinction of a solution of desoxyribonucleic acid (DNA) also at a concentration of 10^{-10} mg per μ^2 as derived from Caspersson [4].

Column 3 lists the average extinction of a typical nucleus from the tip 2.5 mm or a root tip, and column 5 that of one from the second 2.5 mm region. To determine the quantities of the nucleic acid and amino acids that would equal the absorption of these nuclei two simultaneous equations were solved to fit a combination of the solutions of columns one and two to the nuclear absorption at 2650 and 2800 Å. Thus:

Nuclear extinction per μ^2 = Nucleic acid specific ext. × Quantity per μ^2 + Amino acid specific ext. × Quantity per μ^2

or, for the tip nucleus of Table I:

$$\text{At } 2650 \text{ Å} \quad 0.504 = 0.210\,x + 0.123\,y$$
$$\text{and at } 2800 \text{ Å} \quad 0.481 = 0.120\,x + 0.143\,y$$

Solving these equations x, the quantity of nucleic acid per μ^2, is 0.84×10^{-10} mg and y, the quantity of the combined amino acids, is 2.66×10^{-10} mg.

These calculations fitted the solution absorptions to the nuclear absorptions at 2650 and 2800 Å. To check the characteristics of the fitted solutions the extinction coefficients to be expected from solutions of these amounts of nucleic acid and amino acids at the non-fitted wave lengths, 2750 and 2894 Å were calculated to compare with the observed. Of the 90 nuclei scanned 87 showed close agreement between the observed and calculated, the "t" value of the difference between observed and expected absorptions of the 87 nuclei taken together being non-significant. The

three that did not fit showed a minimum at 2750 Å although no such minimum appeared in the calculated absorption spectra. Since statistical analysis showed that these three nuclei did not belong to the same population as the others, they were discarded. In addition, one clearly polyploid nucleus was also discarded.

Since the areas of the nuclei had been determined, the total nucleic acid and tyrosine plus tryptophane content was obtained by multiplying the quantity per μ^2 by the nuclear area. This calculation requires the assumption that the nuclei are shaped as cylinders and so are of equal depth over their whole area. There is, unfortunately, no suitable method at present available for measuring whether or not this is true. If the nuclei are spheres or spheroids, then the total nucleic acid and amino acid content has been computed one-third too high. The major emphasis of this study is on changes in the relative amounts of nucleic acid to protein absorption, a relationship that is measured by the shape of the absorption spectrum and so is independent of nuclear shape. While, therefore, the absolute amounts of nucleic acids and amino acids recorded may be in error, the relative quantities are felt to be accurately represented.

These nuclei contain typically between 2 and 4 nucleoli of varying sizes. It was hoped at the start of the study that it might be possible to analyze the nucleolar absorption spectra also, but ultraviolet scannings failed to show any measureable difference between areas of the nuclei containing nucleoli and those not containing them. The nucleolar absorption has, accordingly, not been separated out, but included in the nuclear absorption. The nuclei of Fig. 1 were photographed at 2650 Å, and two nucleoli are clearly evident in the nucleus to the right in the second row from the bottom. Actually all of these nuclei probably have several nucleoli, for these bodies can easily be seen in all nuclei of stained preparations. The fact that they are not recorded in ultraviolet measurements may result from a masking of nucleolar absorption by a very much higher absorption by other parts of the nuclei.

In order to evaluate the relative amount of nucleolar material in typical nuclei of the mitotic and differentiated areas camera lucida drawings were made at 1700 × of nuclei and their nucleoli in longitudinal sections of root tips. Preparations for this purpose consisted of root pieces from the tip and second 2.5 mm regions sectioned 15μ thick and stained in crystal violet. Nucleolar areas were determined from the camera lucida drawings by planimetric measurements and results expressed as total nucleolar area per nucleus. This total was sometimes all in one nucleolus, but much more typically summed the area of two or more.

RESULTS

The first and most striking feature of these absorption spectra was their variability. Many of the nuclei, particularly those of the mitotic area, were predominated by amino acid absorption, no fewer than 13 of the 39 mitotic area nuclei actually having a higher 2800 Å than 2650 Å absorption. Only 2 of the 47 differentiated area nuclei, on the other hand, showed the 2800 Å maximum, these nuclei having absorption spectra more dominated by nucleic acids. These differences in shape could at the outset indicate either that the

TABLE II

Comparison between the characteristics of nuclei from the mitotic and the differentiated areas of *Tradescantia* root tips. Standard errors indicated show the maximum variation of the mean to be expected in 99 out of 100 trials.

Characteristic	Mitotic	Differentiated	"*t*" of difference
Area in square microns	170 ± 25	248 ± 32	5.00**
Nucleic acid $\times 10^{-10}$ mg per μ^2 . . .	0.99 ± 0.27	1.07 ± 0.19	0.68
Total nucleic acid* $\times 10^{-10}$ mg . . .	183 ± 37	268 ± 62	Chi square against equality 15.02**
Amino acid $\times 10^{-10}$ mg per μ^2 . . .	2.19 ± 0.46	1.26 ± 0.25	5.05**
Total amino acid $\times 10^{-10}$ mg	338 ± 73	284 ± 50	1.70
Total nucleolar area in μ^2	27 ± 4	10 ± 2	19.3**

* The data for total nucleic acid of mitotic acid nuclei showed a Poisson distribution. The fiducial limit for this was accordingly calculated using a Poisson formula, and Chi square was used to compare it to the normally distributed data showing total nucleic acid of the differentiated nuclei.
** Odds of 99:1 or greater against equality of the two compared data.

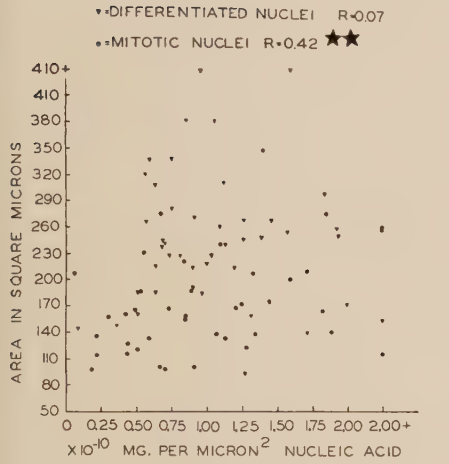
tip nuclei were very rich in protein, or that they were poor in nucleic acids, or some combination thereof.

Three other differences between these populations of nuclei were also immediately evident. The tip nuclei were on the average smaller, a difference that was highly significant (Table II). The comparisons of the two root regions of a number of different tips showed that, as reported by Caspersson [5], a larger nucleolar area occurred repeatedly in the mitotic than the differentiated region nuclei. The nucleolar area data in Table II are derived from measurements of 25 nuclei in each of the two regions of two roots.

Lastly, the extinction values of the mitotic nuclei were generally higher than were those of the differentiated nuclei. These high extinction values suggest that there is a high concentration of absorbing material per μ^2 of the small mitotic nuclei.

The differences in height of the extinction curves, shape of the curves, and nuclear areas indicate that changes in relative and absolute contents were occurring simultaneously as the nuclei grew. Information concerning the quantity changes of nucleic acids and amino acids could, therefore, only be derived from analysis of the absorption spectra.

Nucleic acid content.—The relationship between nucleic acid content per μ^2 of nuclear surface and nuclear area is shown in the scatter diagram of the upper part of Fig. 2. It is apparent here that there is no consistent and striking tendency for these two to be associated. If the nucleic acid content



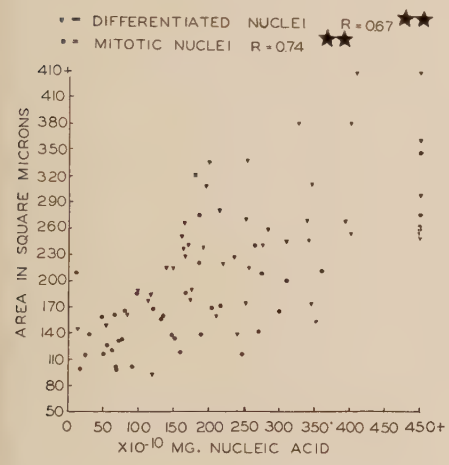
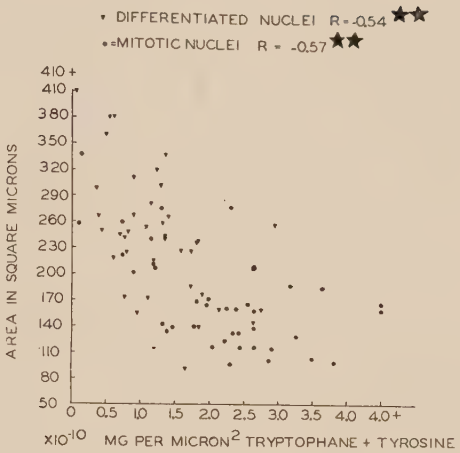


Fig. 2. The upper graph is a scatter diagram showing the relationship between nuclear area and quantity of nucleic acid per square micron of nuclear surface. Below is shown the relationship between nuclear area and the total nucleic acid content of the nuclei. Two stars by the correlation coefficient indicate that the correlation is above the 99:1 level of significance.



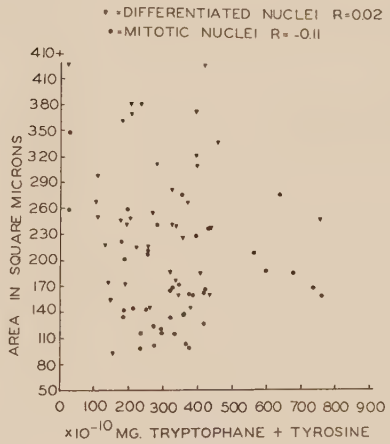


Fig. 3. *Above:* A scatter diagram showing the relationship between nuclear area and quantity of the two amino acids per square micron of nuclear surface. Two stars by the correlation coefficient indicate that the correlation is above the 99:1 level of significance. *Below:* A scatter diagram showing the relationship between nuclear area and total amino acid content per nucleus.

of these nuclei were constant then there should be a *negative* correlation resulting from the fact that large nuclei would have a low nucleic acid content per unit area and small ones a high content. There was, to the contrary, no correlation in the differentiated nuclei and a low but significant *positive* correlation in the mitotic nuclei. There are at least two possible interpretations of this positive correlation. One is that nucleic acid is being formed at a slightly faster rate than that of nuclear growth, so that as the nuclei grow the nucleic acid concentration also increases. Another possibility is that larger nuclei are slightly thicker, and since the ultraviolet light must pass through a thicker nucleus a greater amount per unit surface area is recorded. The low size of this correlation coefficient argues, in any case, against its importance.

The total quantity of nucleic acid per nucleus is significantly correlated with nuclear area (Fig. 2—lower graph). This pattern occurs in nuclei of both the differentiated and mitotic regions, indicating that growth of nuclei of both regions is associated with an increase in their nucleic acid content. The contrast of quantity of nucleic acids in nuclei of the mitotic area with that of those of the differentiated area is shown in Table II. There is no significant difference between amount of nucleic acid per unit area of nuclei of these two regions of the root tip, but since the differentiated nuclei tend to be larger, their total nucleic acid content is significantly greater.

Amino acid content.—As these nuclei increase in area the quantity of tryptophane plus tyrosine present per unit of surface area actually decreases (upper half of Fig. 3). The two highly significant negative correlations show that this pattern applies both to nuclei of the mitotic and the differentiated area. The result of this, as figured below (Fig. 3) is that large nuclei do not have any greater total amino acid content than do the small ones. The large nuclei appear merely to retain the same quantity, but to have it diluted over a greater area. The larger nuclei of the differentiated region accordingly contain the same total amino acid content as do the smaller mitotic area nuclei (Table II).

DISCUSSION

The ultraviolet technique cannot distinguish between ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) unless used in conjunction with enzymes as was done by Leuchtenberger, Klein, and Klein [11] and Frazer and Davidson [7]. These data, therefore, apply to the sum of the two nucleic acids plus any free nucleotides that may be present. The amount of DNA present in nuclei of root tip cells of *Tradescantia paludosa* has been determined

by Swift [22] using the Feulgen staining reaction. He found that the quantity present fell into a continuous series from a value of ca. 8 arbitrary units to one of 16, the lower value occurring at early and the upper at late interphase. This result indicated that the largest nuclei had about twice as much DNA as had the smallest. The smallest nucleus of the present study contained ca. 12×10^{-10} mg of nucleic acid and the largest had ca. 836×10^{-10} mg. While most fall between these extremes, the nuclei represented a total continuous range of values in which the largest had ca. 70 times as much nucleic acid as had the smallest. If results obtained from measurements using these two techniques are comparable, the greater range obtained from the ultraviolet measurements suggests that much if not most of the nucleic acid under consideration is RNA. A study of Stern and Mirsky [21] has shown that RNA is present in certain plant nuclei in relatively high concentrations.

Since is it not possible to be certain that the amino acids are present in the proteins of these nuclei in a constant amount, results have been expressed in terms of tryptophane plus tyrosine. The average protein molecule of diverse bulk plant material contains 2.6 per cent tyrosine and 1.7 per cent tryptophane [14] or a total of 4.3 per cent of the two amino acids. If the assumption is made that these amino acids constitute 4.3 per cent of the protein of these nuclei then the figures listed in the graphs can easily be converted to "Protein per square micron" and "Total protein per nucleus" by multiplying the "Tyrosine plus tryptophane" values given by 23.3. The mitotic nuclei would then contain an average of 79×10^{-8} mg of protein, while the differentiated nuclei would average 72×10^{-8} mg of protein.

The correlation between total nucleic acid per nucleus and nuclear area shows that as the nuclei grow through interphase the total nucleic acid content also increases and, indeed, the nucleic acid may be the agent causing the size increase. By contrast, the content of the amino acids remains constant and they are merely diluted over a larger and larger area as the nuclei grow. Since the amino acid build-up does not occur, apparently, during interphase it must occur some time between prophase and telophase—when the actual stages of cell division are taking place. A study has been carried out of changes in the ratio of nucleic acid to protein of prophase through telophase chromosomes using grasshopper spermatocytes as experimental material [5]. The absorption spectra indicated that chromosomes lost much of their protein during prophase and re-gain it at telophase. This finding suggests that the time of nuclear protein increase in the telophase period immediately after cell division.

The differentiated nuclei were a population of predominately large size

and high nucleic acid content. This fact indicates that the occurrence of differentiation did not result from failure of nuclear growth, and so failure of nuclei to reach the division stage. The differentiated area contains, moreover, nuclei of the different sizes and chemical contents seen among those from the mitotic area, but in a different frequency. There is, accordingly, no set constitution of a nucleus that has stopped dividing. The large size of the majority of differentiated nuclei is likely to be a result of non-division rather than a cause.

SUMMARY

The nucleic acids and protein content of single interphase nuclei of *Tradescantia paludosa* was determined in order to follow the changes that occur during nuclear growth and to compare the content of nuclei from differentiated cells with that of nuclei from cells of actively dividing tissue. The nucleic acids and amino acids used to label proteins were measured by analysis of ultraviolet absorption spectra obtained through the procedures developed by Caspersson. An increase in size as interphase nuclei grow was found to be accompanied by an increase in total nucleic acid content. The total protein content does not increase during interphase growth, so that large nuclei contain the same amount as small nuclei, although diluted, as the nuclei grow, through an increasing volume. It is therefore probable that while the nucleic acid content increases during interphase, the protein content increases during the actual stages of cell division. The nuclei of differentiated cells were on the average larger and contained more nucleic acid, though no more protein, than did those in the area of active cell division.

Contributions to carry out these investigations were given from the Karolinska Institute, Stockholm, Sweden, the Department of Plant Biology, Carnegie Institution of Washington, Stanford, California, and the Department of Botany, Pennsylvania State University, University Park, Pa. The author wishes to express his gratitude to Professor T. O. Caspersson for making available to him the facilities of the Karolinska Institute and for very helpful discussion and advice offered throughout the course of the work. He is also greatly indebted to Dr. G. Moberger, Dr. G. Klein, Mr. Jan Kudynowski, and Mr. Mark Karrel for their help without which the study could not have been completed.

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OUTFLOW FROM CUT ENDS OF NERVE FIBRES

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In excised living nerve fibres the content exudes slowly from the cut ends. This phenomenon was observed by various workers [3, 6, 12, 13] but it has not been studied in detail.

In Young's paper camera lucida drawings of the appearances of the outflow from a giant fibre of *Sepia* at successive intervals of several minutes are shown. The amount of emergent material increases rapidly but the irregularity of its shape prevents an exact evaluation of volumes and rates.

A quantitative analysis of the outflow of contents from the cut fibres is much easier in peripheral nerve fibres of frog. Several features of these fibres make them particularly suitable for such study. (1) The axoplasm of frog is liquid and less easily gelifiable under mechanical stimulation, unavoidable during teasing, than the axoplasm of many mammalian fibres. (2) The liquid axoplasm is completely wetted by myelin which spreads rapidly over the surface of the cross-section. The free borders of the myelin tube coalesce, enclosing completely the newly formed surface of axoplasm. (3) The outpouring material usually forms a perfectly spherical, gradually increasing droplet, composed of an axoplasmic interior and an envelope of myelin. Thus, the total amount of extruded material remains enclosed in a myelin bag and does not mix with the surrounding medium. (4) The boundary of the drop is perfectly visible without fixation and staining and its diameters can be measured under the microscope at various time intervals without interference with the course of the flow. The volume of the exudate and the rate of the flow are easily calculated from these measurements.

MATERIALS AND METHODS

The experiments were performed on carefully teased fibres from sciatic nerves of the frog *Rana esculenta*. The bathing fluid was a Tasaki solution enriched with glucose up to 1 or even 2 per cent. Such excess of glucose in the medium delays the widening of incisures of Schmidt-Lantermann and the swelling of Schwann cells

cytoplasm and ensures a more regular flow. As is reported elsewhere [9], the increased concentration of glucose does not appear to disturb the osmotic balance of the fibre.

In order to avoid compression between slide and coverslip, the teased fibres were examined in chambers about 90μ deep, where they floated freely.

The depth of the chamber is a serious drawback for photography as only small portions of fibres appear in focus at each setting of the microscope. The measurements were therefore made directly on a ground glass projection of the image in an inverted Reichert microscope at a magnification of 1000 ×, and the photomicrographs are reported for illustration only.

The length of the excised fibres was of the order of 1 cm. The normal diameter of the fibre was determined from a mean of several measurements along an uninjured internode. At various time intervals after the excision of the fibre, two mutually perpendicular diameters of the entire drop and two of axoplasm alone were measured. They served for calculation of the volume of extruded axoplasm and myelin. The distance of the cut end from the nearest node, the diameter of the progressively diminishing opening and the diameters of the terminal portion of the fibre at 30, 60 and 90μ from the cut end were also measured in each fibre.

Between measurements the fibres were kept in thermostats at fixed temperatures between 15° and 40° C.

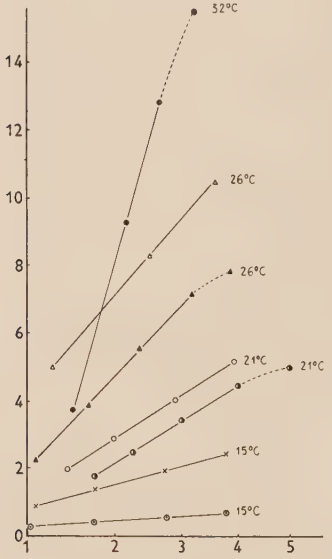
The experiments lasted usually from 8 to 24 hours. In favourable cases the evolution of the fibres could be followed for several days and even for weeks, as the Wallerian degeneration sets in much later in frogs than in mammalian fibres.

RESULTS

The initial appearance of the cut ends.—The cut ends of fibres in a preparation present different appearances according to chance differences in the degree of mechanical injury suffered during the removal of the nerve and during the teasing. In some fibres the terminal portion is crushed by the cut and filled with a mixture of axoplasm and myelin. They usually remain unchanged for days. In other fibres the normal arrangement of axoplasm and myelin is preserved up to the cross-section. The subsequent evolution of these fibres is determined by the physical state of their axoplasm. Frequently the section of the fibre leads to gelation of axoplasm at the site of the lesion. Similar gelation of injured protoplasm was observed by many workers [1, 2, 10, 11]. The cross-section of a gelated fibre remains flat and is not coated by myelin. The gelated portion of axoplasm forms a plug which prevents the flow from the fibre. This plug may be eventually extruded and is then recognizable by its irregular shape (Fig. 13). Sometimes the gelation does not involve the whole surface of the cross-section. A small area remains fluid and the axoplasm exudes through a narrow channel (Figs. 10, 11). In the fibres where the axoplasm is fluid throughout, a convex meniscus is

rapidly formed at the cross-section. The myelin wets the axoplasm and spreads over the newly created surface enclosing it completely [8]. The outflowing content forms a spherical, progressively increasing droplet. All measurements reported below were made on such fibres.

The course of the outflow.—The outflowing droplets appear at both ends of an excised fibre if neither of them happens to be crushed or gelated by



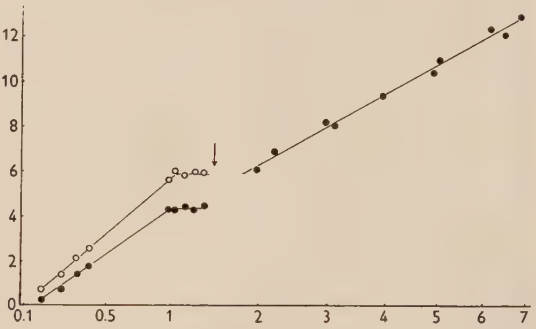


Fig. 2. Time course of the outflow from a fibre before and after the stationary period. Ordinates: volume of emerging content (1000 μ^3). Abscissae: square root of the time (days). Circles: volume of the total drop (axoplasm and myelin). Dots: volume of the axoplasm alone. Arrow shows the moment of bursting of the myelin coating the drop.

Fig. 1. Time course of the outflow from cut nerve fibres of various sizes. Ordinates: volume of the axoplasm (1000 μ^3). Abscissae: square root of the time (hours). Temperature is indicated at each curve. Dashed lines show the period of transition to the stationary state.

the cut. No systematic difference in the outflow from the central and the peripheral ends could be detected, although the original direction of each fibre in the body was easily recognizable by the dissimilar shape of proximal and distal bulbs at the nodes of Ranvier [9].

The time that elapses between the excision of the fibre and the beginning of the outflow varies greatly from one fibre to another. Such variability was observed also in giant fibres of *Loligo* by Flaig [3] who attributed it to a local reversible gelation of axoplasm at the cross-section.

When a spherical droplet of emerging material is formed, it increases gradually for several hours and then the flow is arrested rather abruptly and the volume of the drop becomes for a time stationary. This stationary

period is ended, sooner or later, by spectacular changes in the myelin of the drop. Suddenly the myelin membrane bursts and retracts from the drop towards the orifice of the fibre where it forms a ragged uneven collar (Figs. 7, 8, 12). The drop of axoplasm is left completely naked over most part of its surface. As soon as the myelin is removed from the drop a new spurt of outflow of axoplasm begins.

Sometimes the disruption of myelin does not affect its whole thickness. A layer is broken at the distal end of the drop and glides back to the fibre, whereas another sheet of myelin remains on the drop. In such fibres the further outflow usually leads to the formation of one or several additional droplets besides the initial one (Fig. 6).

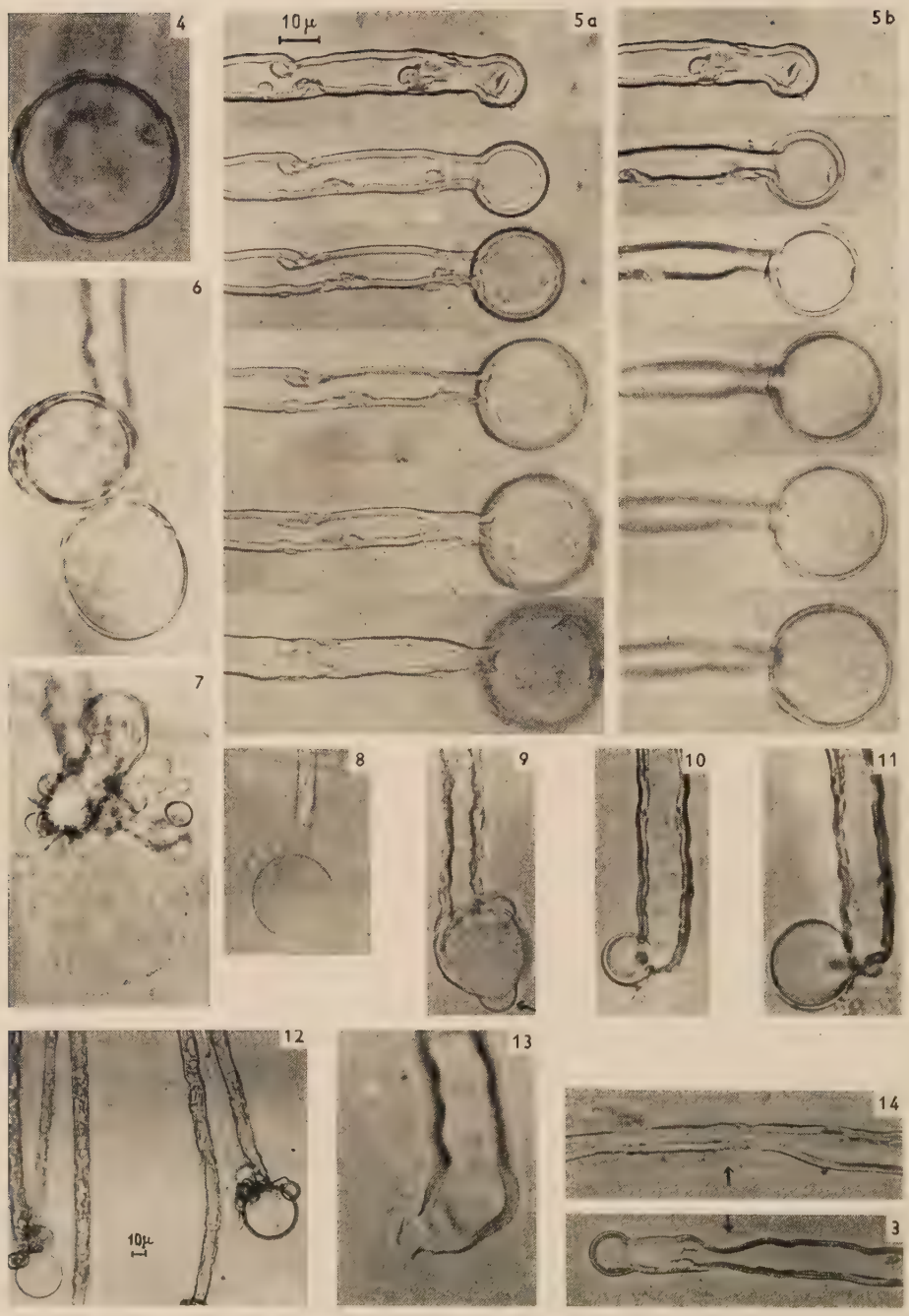
The volume of the discharged axoplasm is proportional to the square root of the time (Fig. 1). This relation is disturbed only during the stationary period and a short time before its beginning, when the rate of flow declines rapidly. After bursting of myelin the measurements of the outflowing axoplasm are often very difficult. In some optically favourable cases it could be ascertained however that the renewed discharge also increases linearly with the square root of the time. Fig. 2 shows the course of the outflow from a fibre which could be measured for over a week.

As is seen in Fig. 1, the rate of the outflow increases with temperature. It depends also on the diameter of the fibre and is influenced by many accidental circumstances such as a narrowing of the lumen of the fibre by an accumulation of Schwann cell cytoplasm near the cut end or by partial gelation of axoplasm at the cross-section.

The fine granules which appear in the axoplasm of ageing fibres do not prevent the flow, and the fact that the further increase of the drop does not alter its spherical shape indicates that the matrix of axoplasm remains fluid.

The terminal portion of fibres during the outflow. The shape of the terminal portion of the fiber is progressively altered during the outflow. The change starts at the cut end where the diameter of the fibre diminishes. This reduction of diameter spreads very slowly up the fibre but remains most marked at the cut end, where the diameter of the fiber is finally reduced to about 0.4 of its initial value. The change of the shape of the terminal portion of a fibre during the outflow is shown in photomicrographs of Fig. 5*A*, the simultaneous increase of the outflowing droplet in Fig. 5*B*.

The Schwann cell cytoplasm does not flow. When the cross-section of the fibre happens to fall near an accumulation of Schwann cytoplasm, it is seen to remain motionless while the axoplasm flows through the partly obstructed lumen of the fibre (Fig. 14).


4
10 μ
5a
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3

DISCUSSION

As the outflow of content described in the present paper occurs in fibres separated from their perikarya, it has to be attributed to forces generated locally along the fibres by interaction of their various components. It was shown in earlier papers [6, 9] that in a normal nerve fibre the neurilemma is distended by the turgor pressure of axoplasm and that this deformation is reversible. The diameter of a fibre is determined by an equilibrium between the elastic tension of the neurilemmal tube and the pressure exerted by the content of the fibre. The diameter diminishes when the intraaxonal pressure decreases and expands with the increase of pressure.

When a nerve fibre is opened by the cut, the pressure at the external surface of the cross-section drops to zero and the gradient of pressure thus established initiates the outflow.

Under the curved surface of the incipient droplet a pressure is built up by the surface tension of axoplasm. It must retard the outflow initially, but an accurate evaluation of its role is impossible owing to many complicating factors intervening immediately after the cut. This pressure decreases with the increasing radius of curvature and, by the time when a sufficient amount of axoplasm has emerged to form a spherical drop, the impeding influence of the pressure due to surface tension seems negligible.

It is from the time of appearance of the spherical drop that the outflow is studied quantitatively in the present experiments. The emerging droplet

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Fig. 3. Outflowing drop, 2½ hours after section. Arrow—Schwann cell cytoplasm.
Fig. 4. Stationary drop, 25 hours after section. Incisures are seen in the myelin envelope.
Fig. 5. Successive stages of the outflow at 1, 1½, 2½, 4, 5 and 7 hours after section. 5 *A*—focussed on the terminal portion of the fibre to show progressive reduction of the diameter. 5 *B*—focussed on the outflowing drop.
Fig. 6. Two drops formed at the end of a fibre 27 hours after cutting.
Fig. 7. Naked sphere of axoplasm. Broken myelin membrane retracted in irregular folds near the cut end, 36 hours after excision.
Fig. 8. Similar naked drop 24 hours after excision. Axoplasm is homogeneous.
Fig. 9. Beginning of a new outflow (arrow) from a drop after disruption of myelin at its distal end. Nine hours after excision of the fibre.
Figs. 10 and 11. Flow from a fibre partly obstructed by a gelated plug. Two and 3 hours after cutting. The myelin is thinner on the drop than in the fibre. Numerous myelin figures (out of focus) near the end of the fibre.
Fig. 12. Low power photograph to show the late appearance of the cut ends, 36 hours after cutting. Collars of retracted folds of myelin near the ends. Left hand fibre—naked drop of axoplasm. Right hand fibre—a thin layer of myelin remains on the drop.
Fig. 13. Gelated plug at the end of the fibre. A portion of naked axoplasm is extruded. Myelin figures near the end.
Fig. 14. Swollen Schwann cell cytoplasm (arrow) partially occludes the lumen of the fibre leaving a narrowed path for axoplasm and myelin.

increases progressively and the volume of the discharged material is proportional to the square root of the time. Such time course of the outflow from distended elastic capillaries was predicted on analytical grounds by Hermans [5] and was confirmed experimentally by the study of the flow from the latex tubes in tapped bark of laticiferous plants by Frey-Wyssling [4]. Simultaneously with the increase of the outflowing drop, the shape of the terminal portion of the fibre is altered. The diameter of the orifice diminishes and, with time, progressively increasing length of the fibre becomes depleted and reduced in diameter.

As it was shown elsewhere [7], the amount of myelin available for coating the drop is limited and when it is exhausted by the increased surface of the expanding drop, the myelin, which is relatively inextensible, becomes strained and exerts tension which increases the pressure inside the drop. Thus, when the emerging drop approaches a definite magnitude, the pressure in the drop counterbalances the pressure driving the content out from fibre and the outflow is arrested.

During the stationary period, the equilibration of pressures between the terminal, partially depleted portion of the fibre and its more remote parts not yet involved in the flow, goes on. As the outflow is prevented, this leads to an increase of pressure in the terminal portion of the fibre and in the drop communicating with it directly. Finally, when this pressure exceeds a critical value, the myelin envelope is broken, leaving an unobstructed path for further discharge.

SUMMARY

When the axoplasm of isolated nerve fibres of frog is fluid, the content emerges slowly from the cut ends of the fibres, forming a spherical droplet of axoplasm invested by a layer of myelin.

The volume of the discharged axoplasm is proportional to the square root of the time.

The terminal portion of the fibre narrows during the outflow. The reduction of diameter is most marked at the cross-section and fades gradually away with the increasing distance from the cut end.

When the outflowing drop reaches a definite magnitude, the flow is arrested and the volume of the drop becomes for a time stationary. The stationary period is ended when the myelin membrane coating the drop bursts. The outflow is resumed immediately afterwards.

The mechanism of the outflow from cut nerve fibres is discussed.

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NUCLEOPROTEIN FRACTIONS FROM VARIOUS EMBRYONIC TISSUES

A COMPARISON OF PHYSICOCHEMICAL CHARACTERISTICS AND BIOLOGICAL ACTIVITY IN TISSUE CULTURE[1]

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Chick embryo extract used to stimulate growth in tissue culture has been found to possess two major active constituents [2]: a dialyzable portion, and a nondialyzable one from which the active ingredients were isolated in the form of a nucleoprotein fraction (NPF) [7]. In this study we consider only the latter active fraction. The nucleoprotein fraction (NPF) of 12-day chick embryos was found to be selectively precipitated with streptomycin [7]. Chemical analysis disclosed that this fraction contains approximately 10 per cent ribonucleic acid, and ultraviolet spectrophotometry revealed a typical nucleoprotein curve with maximum at 260 mμ [7]. This fraction exhibited four electrophoretic components, some of which we have recently demonstrated in preliminary experiments [7] to be inactive in tissue culture. When further methods of purification were considered, the heterogeneity of embryonic tissues and organs suggested that a preliminary dissection of the embryos into tissue and organ batches and subsequent isolation of various tissue NPF's might result in purer products and would perhaps segregate the biological activity.

Previous workers had tested saline extracts of chick embryo tissues and organs in culture, but their results are not necessarily applicable to this study because of the above-mentioned low-molecular-weight dialyzable fraction. Correlations of high activity with saline tissue extracts would not necessarily indicate a high content of NPF, but a correlation of low activity

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would indicate that both NPF and low-molecular-weight materials were present in lesser amounts. Hueper [6], working with 8-day chick embryos, found the eyeless head, eyes, and body to have the same potency as the whole-embryo extract. Fowler [4] compared extracts of 14-day chick embryo head, neck, and trunk with the whole extract; the trunk proved least potent. Fischer [3], working with 7- to 10-day embryos, found the eyes, brain, and internal organs to be less potent than whole-embryo extract. Davidson and Waymouth [1] found sheep embryo cartilage to have a potency similar to the whole 9-day chick embryo extract. These results suggest that perhaps certain regions of the embryo contain more active materials than others.

It was therefore decided to isolate and test the tissue and organ NP fractions, then to analyze by electrophoresis with subsequent pipetting of subfractions and ultraviolet-spectrum analysis of these subfractions.

METHODS

The following tissues and organs of 12-day chick embryos were cleanly dissected and placed in separate batches on an ice bath prior to chemical isolation: muscle (breast and thigh), liver, lung, kidney, gut (upper and lower), heart, brain, eyes, carcass, cartilage (femur), skin, and spine. A few whole embryos were set aside to furnish whole-embryo control material. The NPF was obtained by use of the streptomycin precipitation method [7] with minor modifications. The final products (approx. 30 to 40 cc) were dialyzed overnight against 7 liters of Gey's solution prior to culture operations, and spun at 105,000 g to reduce contaminating microorganisms to a low level, as previously described [7]. Culture methods were similar to those described previously [1, 8, 9].

Fresh explants of 12- to 14-day chick embryo heart, about 1 mm^3 were cultivated in carrel flasks at 37°C for 8 days. The basal assay medium consisted of a chicken plasma clot with a supernatant containing 30 per cent horse serum and 70 per cent Gey's saline. Antibiotics were routinely incorporated into the media. Final concentrations of NPF used in culture were approximately 0.3 mg/cc, i.e., 0.7 mg/culture, on a dry-weight basis. All tissues and organs within a series were cultured at similar concentrations of NPF, and two control sets of cultures were carried through in each test series, one (minimum control) containing Gey's solution and the other (maximum control) containing whole-embryo NPF at approximately the same concentration as the tissue and organ NP fractions. Media were changed at 4 days, and cultures were traced and terminated at 8 days. The areas were determined and statistics calculated as in a previous paper [9]. A Klett moving-boundary apparatus was used for electrophoresis. The conditions for the runs were: 0.1*M* potassium phosphate buffer, pH 7.2; 7.4 ma, giving a field strength of 2.9 v/cm; 0.5°C water bath temperature; 110-minute duration of run. Samples of subfractions were pipetted in order from top to bottom with a capillary pipette. A Model DU Beckman spectrometer was used for ultraviolet spectrophotometry.

RESULTS

Effects of the nucleoprotein fractions in tissue culture.—The measured 8-day areal outgrowth elicited by the tissue and organ NPF's from the 12-day chick embryo was analyzed statistically for each of the three experiments. The actual areal increase, standard errors, and probability of chance occurrence are given for a sample experiment (March) in Table I. The increases in these three experiments relative to their respective serum controls are comparable on a probability basis; the combined data are shown on a histogram, Fig. 1, illustrating the 1 per cent level of insignificance—i.e., 99 per cent probability of significant difference—often used in tissue culture and chosen for this work. The three NP fractions that are as effective as whole-embryo NPF comprise the "highly active" group. For comparative purposes the 3 per cent and 5 per cent levels of insignificance are also shown. In simultaneous experiments at a different laboratory, one of us (R. C.) tested the various NP fractions in roller tube cultures and obtained essen-

TABLE I

Sample (March series) effects of embryonic tissue and organ NP fractions on 8-day outgrowth of chick heart fibroblasts.*

Tissue NPF	Area increase** (mm^2)	S. E.**	P† %	$100-P$†† %
Serum controls (basal medium) . . .	29.4	10.2	—	—
Heart	48.6	7.3	19.8	80.2
Skin	52.4	5.4	8.6	91.4
Kidney	48.9	7.1	16.0	84.0
Liver	38.4	4.0	45.0	55.0
Gut	54.4	2.8	5.0	95.0
Lung	62.2	1.8	2.5	97.5
Muscle	40.9	7.0	39.0	61.0
Brain	48.2	2.5	11.5	88.5
Carcass	82.9	4.4	0.19	99.81
Cartilage	85.2	7.1	0.27	99.73
Spine	75.4	5.6	0.76	99.24
Whole	72.6	3.2	0.98	99.02

* Medium: Chicken plasma clot, 30 per cent horse serum, 70 per cent Gey's solution tissue NPF at 0.30 mg/cc in Gey's solution.

** Mean value and standard error, 4 to 5 cultures per series.

† Fischer's t-test (Snedecor [10]) P = probability that areal difference relative to serum controls would occur by chance alone.

†† Probability of difference from serum controls = $100-P$.

tially similar results. The microscopic appearance of the cultures with areas equal to or larger than whole-embryo NPF was comparable to that of the healthy appearance of cells cultured in whole-embryo NPF as previously described [7], thus furnishing parallel evidence of effectiveness of the various NP fractions.

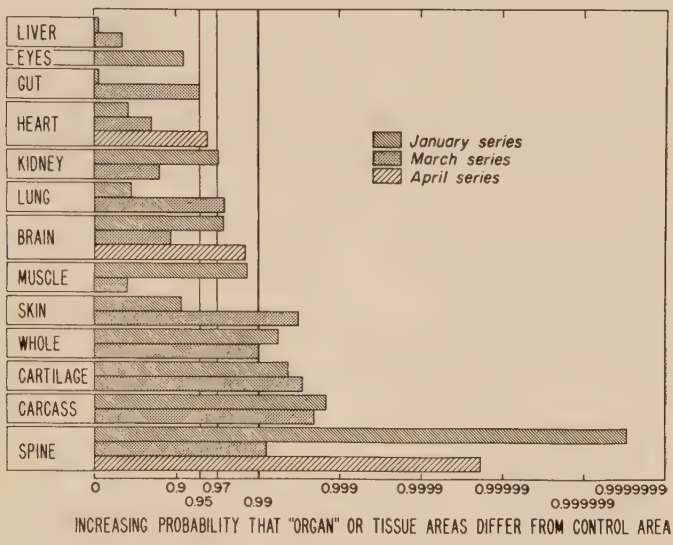

Fig. 1. Combined data from January, March, and April series.

Electrophoretic patterns of the nucleoprotein fractions.—The electrophoretic patterns for the NP fractions obtained from the various tissues and organs showed from two to four distinctly discernible maxima for each NPF, exclusive of the salt boundaries, (two in cartilage, four in gut, eye, heart, whole and carcass, three in the other tissues except kidney, which was not done). For the March series, for example, Fig. 2 *A* shows the photographic ascending and descending patterns obtained from gut, and Fig. 2 *B* those from cartilage. On the basis of three separate dissection series it appeared that each tissue or organ yielded a reproducible and characteristic pattern.

The average patterns of the "highly active" and "other" tissues traced to the same scale of total area are compared in Fig. 3. Since the whole and carcass contain mixtures of both groups of tissues and organs, they are not included in the average of the highly active fractions.

Fractions of the *whole-embryo* NPF corresponding to various areas on the schlieren pattern were pipetted after electrophoretic separation and tested in tissue culture. The preliminary finding [8] of an active subfraction

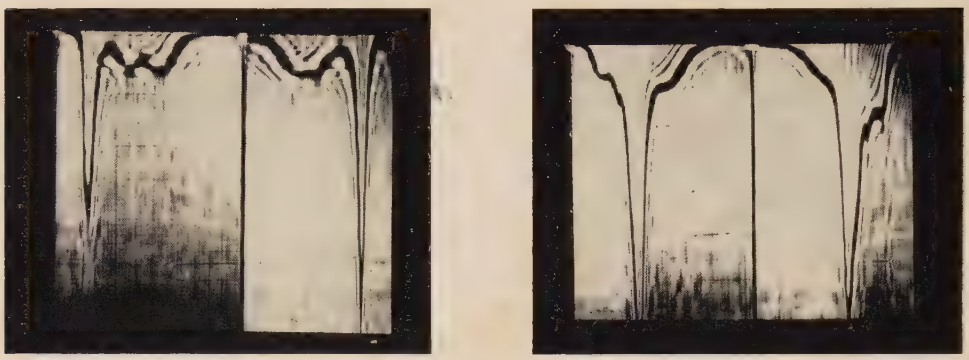

Fig. 2 *A* (left). Photographs of electrophoretic patterns of gut NPF. Ascending on right.
Fig. 2 *B* (right). Photographs of electrophoretic patterns of cartilage NPF. Ascending on right.

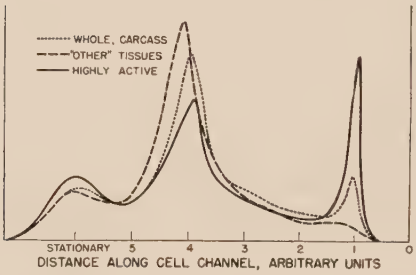

Fig. 3. Comparison of average electrophoretic NPF patterns (March, ascending limb).

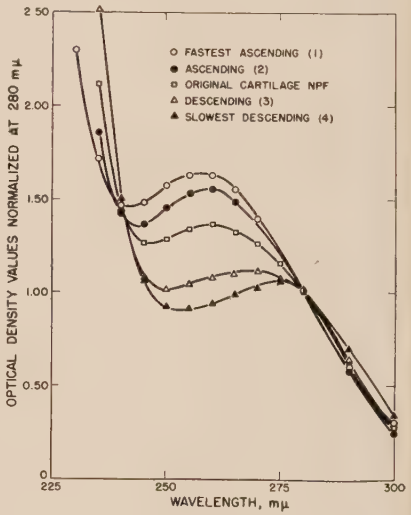

Fig. 4. Ultraviolet spectra of pipetted subfractions. January cartilage NPF electrophoresis.

was confirmed in that the activity seemed to be concentrated in an area of intermediate mobility, namely between 2.5 and 4 units on Fig. 3.

Ultraviolet absorption spectra of the nucleoprotein fractions.—Each of the NP fractions showed a nucleoprotein-type U.V. absorption spectrum with a maximum at 260 mμ, which was very similar for all organs and tissues and resembled the spectrum of the original whole-embryo NPF [7].

Ultraviolet analyses were performed on various subfractions of tissue NP fractions pipetted from the moving-boundary electrophoresis. All the subfractions were found to exhibit similar families of curves in which the

maximum shifts gradually, in going from the fastest to the slowest subfraction, from 257 mμ to 275 mμ. For example, cartilage is shown, Fig. 4, values normalized to 280 mμ, in 0.1*M* phosphate pH 7.2.

DISCUSSION

The histogram in Fig. 1 indicates that only extracts of spine, cartilage, and carcass have consistently had an activity in terms of areal increase equal to the whole embryo extract. These results might implicate extracts of the major common constituent, cartilage, as being a potent source of activity in the 12-day chick embryo NPF. Other tissue extracts also contain active material, but in smaller concentration or lower unit activity. These results harmonize with those obtained on saline extracts of chick embryos by Fischer [3] and especially those of Davidson and Waymouth [1] who found sheep embryo cartilage extract to be a potent growth factor. Differences between cartilage and spine may possibly be due either to factors extracted from the spinal cord or to a difference between the stage of development of the femur cartilage and that of the spinal cord cartilage.

Various tissue NP fractions also show definite differences in electrophoretic pattern. The more active fractions exhibit a much greater leading peak than the other fractions. The close reproducibility of the tissue electrophoretic patterns from month to month argues well for the reproducibility of the streptomycin method. The only biologically (*in vitro*) active subfraction of the whole-embryo NPF corresponds to a zone on the electrophoretic pattern that is poorly defined. It would therefore appear that the active compounds in this active subfraction are either polydisperse or are masked on the electrophoretic pattern by other inactive components. It should be noted that none of the tissue NPF electrophoretic patterns exhibits peaks in a zone corresponding to the active zone in the whole-embryo NPF pattern; furthermore the areas under the curves (i.e., total amounts of dissolved material) in the various corresponding active zones are about the same when the more active tissue NP fractions are compared with the other NP fractions. This might indicate either that each tissue NPF has a different constitution and potency or that varying amounts of the same active material are present, mixed with inactive material. The presence of inhibitors might complicate the picture, but this possibility should not be excluded.

The first ascending electrophoretic component of the whole-embryo NPF has a high content of nucleic acid-like materials, as shown by its ultraviolet spectrum, but is inactive. An increase of this first peak is noted when the

NPF is denatured, aged, or further processed. It is not clear why the more active NP fractions possess an enhanced leading peak which presumably is inactive, but perhaps the more active NP fractions are more labile and release their nucleic acid moiety more rapidly than the less active NP fractions.

SUMMARY

1. A nucleoprotein fraction (NPF) was isolated by streptomycin precipitation from twelve different chick embryo tissues.

2. Bioassays in tissue culture revealed maximum growth-promoting activity in those NPF's from material containing cartilage.

3. Electrophoretic analyses indicate that the leading peaks of the highly active NPF's are much greater than those of the other NPF's.

4. Ultraviolet analyses of tissue NPF's indicate similar nucleoprotein absorption spectra, while the electrophoretic subfractions of these NPF's exhibit families of curves which are similar and have maxima from 257 mμ to 275 mμ.

The authors wish to acknowledge the interest shown by Drs. Hardin Jones and Lola Kelly and the special facilities arranged by Dr. Jones for this work. We also wish to acknowledge the technical assistance of Lawrence Adams, Jon Garrett, Laurence Resseguie, and George Okawachi.

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ELECTRON MICROSCOPIC OBSERVATIONS ON GRANULES AND FILAMENTS (RETICULOSOMES) OF RETICULOCYTES

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THERE is no uniformity of opinion about the nature of micro-structures observed in reticulocytes and it still remains to be cleared whether granules and filaments visualized in the optical microscope are pre-existent or may be attributed to the action of stains, fixatives or hemolytic solutions. Braunsteiner and Bernhard [2] could not detect filaments in reticulocytes examined in the electron microscope following hemolysis made with blood suspended in distilled water, but only granules ranging in size from 0.1 to 0.5 μ in diameter.

In this paper electron micrographs are presented showing filaments, granules and circular forms of reticulocytes hemolysed by various methods.

MATERIAL AND METHODS

Hemolysis was made in suspensions or thin smears of heart or peripheral blood [18] obtained from guinea-pigs of approximately 350 g body weight 4 to 7 days after bleeding of 6 ml. Controls in the optical or phase-contrast microscope were made with preparations stained with Janus green.

In suspension.—Immediately after bleeding, 1 ml of blood was added to 40 ml of distilled water (technique *A*) or to 40 ml of 0.2 per cent saline containing 2.5 per cent of formalin (technique *B*). Five minutes later 2 ml of 1 per cent osmium tetroxide solution were added in both cases and centrifuged at 500 r.p.m. after another five minutes. Washings were made until a colorless supernate was obtained. Drops of suitable suspensions of stromas were placed on collodion-coated metal grids and some shadowed with chromium.

In thin blood smears.—Smears prepared on collodion-coated glass slides were allowed to dry in the air for ten minutes and subsequently immersed for five minutes in 0.8 per cent saline containing 2.5 per cent of formalin (technique *C*), in 14 per cent saccharose solution (technique *D*) or in distilled water after drying for 48 hours (technique *E*). Control of hemolysed smears was made by phase-contrast microscopic examinations. The stromas were then stained in 0.5 per cent aqueous osmium tetroxide solution for ten minutes or successively in 1 per cent phosphotungstenic acid solution buffered to pH 7.0 and 4.0 for five minutes, respectively, washed in

distilled water and dried at 37°C. The films carrying the stromas were transferred to metal grids and some shadowed with chromium.

A Siemens electron microscope type UM 100b was used throughout, with tensions ranging from 40 to 60 kV.

RESULTS AND CONCLUSIONS

In reticulocytes of blood hemolysed while suspended in distilled water (technique *A*) only circular forms ranging from 0.3 to 1.0 μ and granules from 0.15 to 0.25 μ in diameter (Fig. 1) were observed. No filaments were found. Of the circular forms twenty or more may be seen inside of one reticulocyte, which appear as if consisting of a flat membrane similar to a small erythrocyte stroma, by which the impression is gained that a globular element was flattened by loss of substance.

The majority of reticulocytes hemolysed in suspension according to technique *B* showed irregular shaped electron-dense masses sometimes presenting not individualized circular forms on its periphery, which in both dimension and aspect are similar to those found in reticulocytes hemolysed according to technique *A* (Fig. 2). Also granules were found in these reticulocytes.

Reticulocytes from blood smears hemolysed according to technique *C* evidenced more or less uniformly distributed granules ranging from 0.15 to 0.20 μ in diameter, which sometimes appear in large number that may even exceed one hundred (Fig. 3). Long filaments of sections ranging from 0.15 to 0.30 μ and of variable length up to 10 μ or more are observed (Figs. 3, 4 and 5) generally attached to an irregular shaped mass mostly in central position within the stroma. Sometimes they are found radially disposed (Fig. 4) or convoluted (Fig. 5) consisting of one long continuous filament practically equal in diameter throughout its length. In some instances it can be observed that both filaments and irregular mass are composed of denser spherical, oval or cylindrical forms surrounded by a less dense continuous region. Both granules and filaments vary considerably in quantity from one reticulocyte to another, which is probably correlated with the actual stage of development. In some filaments the free end appears enlarged, which is well depicted in Fig. 3.

In a case of acute myeloid leukemia De Robertis [3] found in electron micrographs of hemolysed erythrocytes filaments similar to those depicted in Fig. 5, which he suspects not to be correlated with the leukemia but to represent a coexisting element.

The filamentous reticulosomes resemble in both aspect and structure

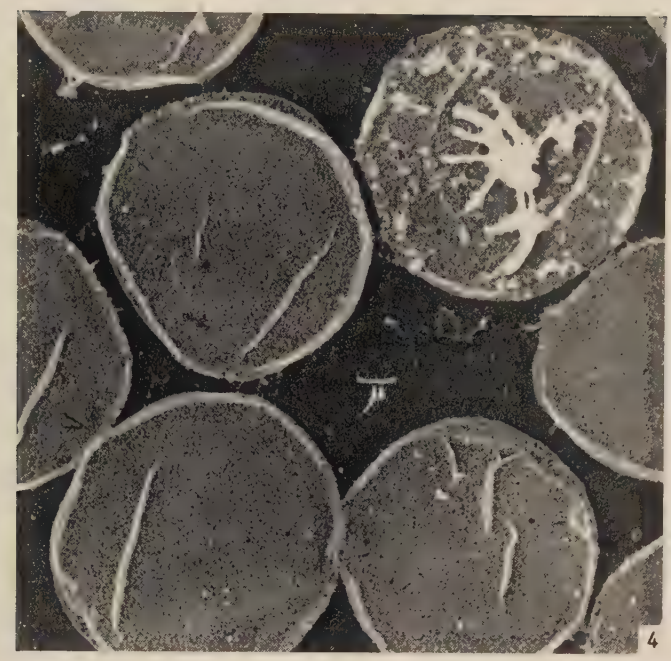
Fig. 1. Electron micrograph of a reticulocyte hemolysed in suspension in distilled water. Shadowed with chromium.

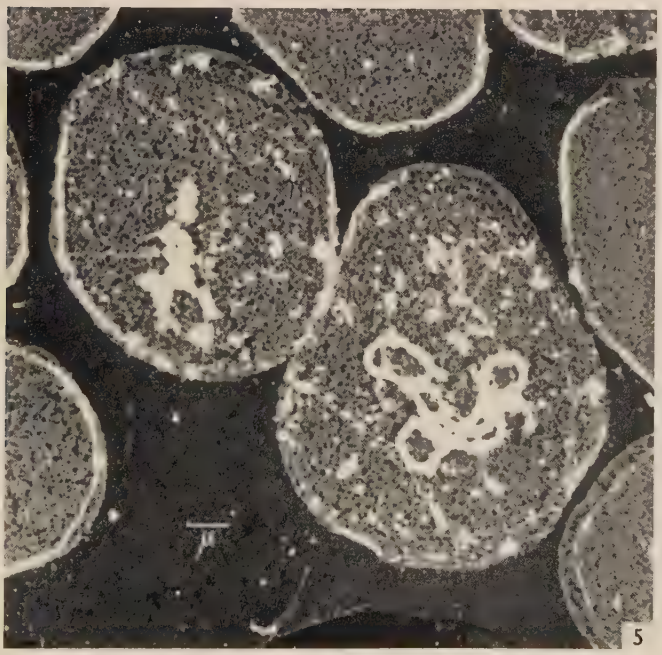
Fig. 2. Electron micrograph of a reticulocyte hemolysed in suspension containing 2.5 per cent of formalin. Shadowed with chromium. The arrow indicates a not individualized circular form.

Fig. 3. Electron micrograph of a reticulocyte hemolysed in 0.8 per cent saline containing 2.5 per cent of formalin after partial drying. Shadowed with chromium.

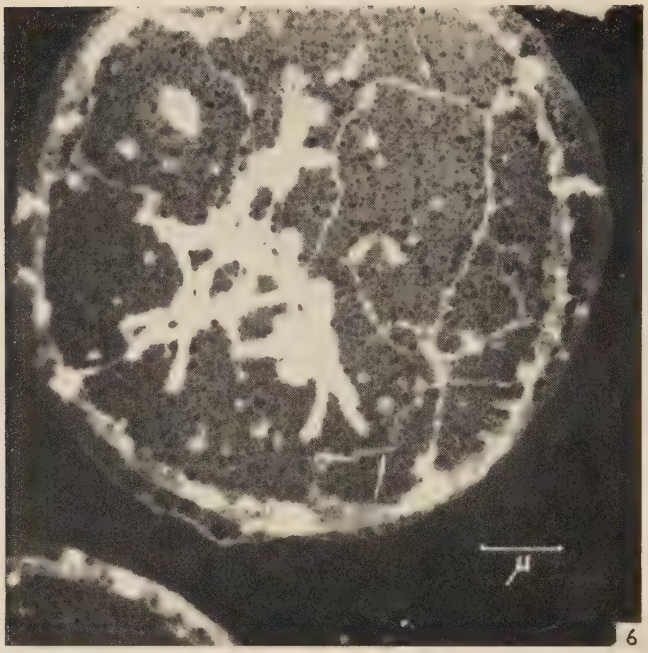
those recently referred to by Porter [7] as the so-called endoplasmic reticulum in cells grown *in vitro*, varying considerably in shapes and dimensions.

Much finer filaments were distinctly observable in non-shadow-cast reticulocytes, some of which presented small granules disposed along their





Figs. 4, 5 and 6. (Fig. 6 on next page.) Electron micrographs of reticulocytes hemolysed in 0.8 per cent saline containing 2.5 per cent of formalin after partial drying. The reticulocytes of Figs. 5 and 6 have not been shadowcast. In Fig. 6 arrows indicate the finest filaments.



length (Fig. 6). None of the circular forms found in reticulocytes hemolysed according to technique *A* (Fig. 1) could be detected in these preparations.

In reticulocytes hemolysed by technique *D*, as well as in those treated according to technique *E*, 48 hours following preparation of smears the presence of filaments was also stated.

Within the same material the frequency of reticulocytes appeared not to vary after vital staining when observed in the electron, the phase-contrast and the optical microscope.

By means of electron microscopy four different forms of reticulosomes were distinguished, namely, (a) granules, (b) circular forms, (c) large filaments, and (d) fine filaments.

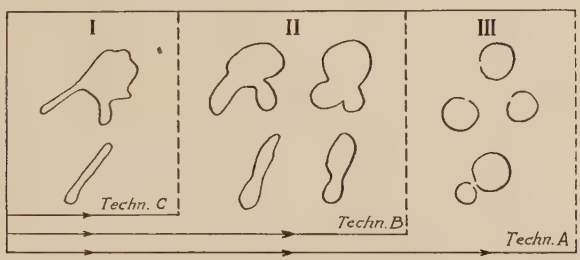
The more persistent element of these four, whatever hemolytical technique is employed, is the granule. It seems that it suffers the influence of low tonicity of the medium, since it appears flatter (Fig. 1) following hemolysis in suspension in distilled water, with diameters ranging from 0.15 to 0.25 μ, while after hemolysis achieved according to technique *B* or *C* it presents itself spherical and denser, measuring from 0.15 to 0.20 μ in diameter (Figs. 2 and 3, respectively).

In correspondence to the hemolytical conditions, there were observed in

the reticulocyte free filaments attached to an irregular shaped mass or only circular forms. It can be assumed that without previous partial drying (technique *A*) filaments undergo disintegration during the hemolytical process in distilled water and thus change into the circular forms. This would be in accordance with the great lability of the chondriosomes that are possibly in the stage of coacervation. The disintegration process can be avoided by the presence of formalin, when hemolysis is made without previous partial drying (technique *B*), and the lability is decreased in hemolysis after prolonged drying (technique *E*).

Lewis and Lewis [6] observed that filamentous chondriosomes in isotonic medium change to globular forms when transferred to a hypotonic medium and become filamentous again, but finer, in hypertonic medium.

Based on the above observation and the aspect of reticulosomes observed after hemolysis as achieved according to techniques *A*, *B*, and *C*, the following mechanism may be suggested:



Reticulosomes

The shapes depicted in I represent forms of the reticulum, which persist when hemolysis is made according to technique *C* (Fig. 3). With technique *B* (Fig. 2) the initial form undergoes changes, thus producing shapes corresponding to those presented in II. The round shapes of III are obtained in blood hemolysed in distilled water without previous partial drying (technique *A*, Fig. 1), passing through all intermediary forms.

This interpretation disagrees with the observations of Kosenow [5], who found through the fluorescence microscope that young erythrocytes stained in increasing acridinorange and H-concentrations showed increasing quantity of granules and filaments, which he believed to depend on the presence of nucleic acids existing in diffuse form. Dustin [4] ascribes the formation of granules and filaments to the presence of ribonucleoprotein and Bessis [1] holds that the basophile substance does not pre-exist in granular form, but is an agglomeration of granules formed by the influence of vital stains.

The observations presented in this paper suggest the pre-existence of integrant elements in reticulocytes. However, the final decision will depend on future investigations using techniques by means of which difficulties thus far encountered may be obviated. Also the knowledge of the chemical composition of the various forms of reticulosomes would help clarifying the problem.

SUMMARY

Electron micrographs are presented showing different forms of integrant elements in reticulocytes hemolysed by various methods, for which the general denomination of *reticulosomes* is suggested. A mechanism is proposed explaining the changes filaments undergo when hemolysis is made in suspension in distilled water.

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ALKALINE PHOSPHATASE IN THE GOLGI SUBSTANCE OF INTESTINAL EPITHELIUM

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The existence of the Golgi apparatus in the living cell has been very much discussed of recent years and the classical picture has even been discarded as a pure artefact [17]. In contrast to this Benseley [2] and Elftman [8] have brought forward some evidence in favour of the opposite opinion. The electron microscope has now revealed that the Golgi apparatus in several tissues is a well-defined morphological unit. This is the case with the intestinal epithelium [4, 5, 18]. The basis for histochemical studies on the Golgi apparatus of the intestinal epithelium is therefore well grounded.

The presence of alkaline phosphatase in the Golgi region of some epithelial cells has been reported by several authors [3, 6, 7, 9, 10, 14]. Interest has chiefly been drawn to the intestinal epithelium and several suggestions on the physiology of the enzyme in general and in this special localization have been made. However, some critical remarks on the histochemical procedure have rendered the interpretation of the histological picture very uncertain and difficult [13, 15]. With regard to the Golgi region, however, Novikoff *et al.* have tried to rule out the possibility that diffusible calcium phosphate causes a false reaction [16]. Nevertheless, diffusion of the enzyme cannot be disregarded even if it seems to be of less practical importance.

In order to reduce the diffusion phenomena a modification of the Gomori method [12] for demonstration of alkaline phosphatase has been suggested [11]. It was demonstrated that artefactual nuclear staining does not occur with this modified method. On the other hand it could not be positively stated that the activity of the Golgi region is genuine, as the "diffusion range", not reaching the nuclear zone, might be large enough to admit of artefactual staining of the Golgi region. Therefore the cuticular border was separated from the rest of the cells in order to exclude the high activity of the cuticular border as a source of diffusible enzyme and reaction product.

MATERIALS AND METHODS

Frozen-dried rat duodenum was used in this study. Dry paraffin sections were employed without any contact with water. After deparaffination in xylene for 3 minutes

the tissue was fixed in absolute acetone for one minute. It was then manipulated with glass instruments in a Singer micromanipulator under phase contrast. Separated tissue was continually washed away by 70 per cent acetone in water at which concentration the enzyme is insoluble (cf. [1]). The arrangement is shown in Fig. 1.

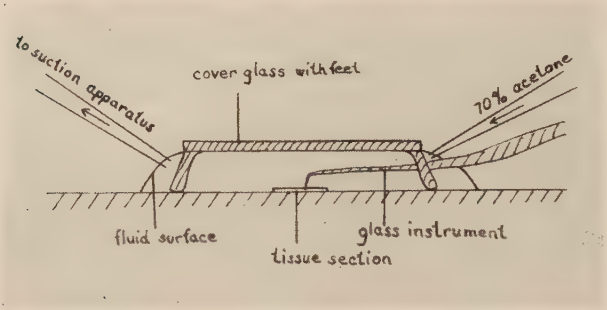

Fig. 1. Schematic figure showing the arrangement of manipulatory instruments.

When the cuticular border was separated from a suitable part of the section, as a rule situated at the base of a villus, all the surrounding remains of the tissue were carefully scraped away. The preparations were then incubated one by one in the original [12] or the modified mixture [11] for different periods of time and the precipitate thus formed was visualized with the cobalt technique. Magnesium chloride was employed instead of sulphate in the incubation mixture to exclude the possibility of calcium sulphate precipitates. Ultraviolet irradiated controls were used.

Fig. 2 (left). Rat duodenum, frozen-dried. Manipulated in 70 per cent acetone. Incubated for 10 minutes. Author's modification. Cobalt technique.
Fig. 3 (right). Rat duodenum, frozen-dried. Manipulated in 70 per cent acetone. Incubated for 10 minutes. Gomori mixture. Cobalt technique.

RESULTS

After successful manipulation phosphatase activity was demonstrated in the Golgi region without any other site of equal or stronger activity being present (Figs. 2–3). There was no significant difference between preparations incubated for 10 minutes in the original Gomori-mixture and the modified mixture. After one hour's incubation the picture was blurred with the Gomori technique but not with the modified one. In preparations where the manipulation was less successful and some parts of the cuticular border were left, it was observed that the staining of the Golgi region was more prominent in the vicinity of the cuticular border than at some distance from it. This shows that the diffusion is only reduced in the modified technique and that diffusion products can add to the products of a genuine activity thus giving the impression of a stronger activity than is actually present.

SUMMARY

To exclude the possibility that the histochemical picture of alkaline phosphatase in the Golgi region of intestinal epithelium is due to a diffusion artifact an experiment was performed. By microdissection the cuticular border of the epithelial cells was removed. Subsequent demonstration of alkaline phosphatase showed a reaction in the Golgi region only. The genuineness of the reaction in this localization is thus established.

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ON THE NUCLEOLUS-ASSOCIATED CHROMATIN

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Two components have been distinguished in the interphase nucleus: the true nucleolus or plasmosome, and heterochromatic granules or chromocenters. The former is Feulgen-negative while the latter are Feulgen-positive. The nucleoli of some insects, however, are not purely Feulgen-negative, but are considered to have heterochromatic material incorporated within them [1]. Davidson [4] has observed in the nucleolus of mammalian liver cells an outer zone of Feulgen-positive material. These observations suggest that the nucleolar structure may be more complex than originally believed.

Hughes [5] has described the chromocenters as small heterocyclic segments of chromosomes which have not lost chromatin at telophase and thereby remain Feulgen-positive. Vanderlyn [9] has reported that they may stick either to each other in aggregates or to the nuclear membrane and the nucleolar surface.

The chromocenters related to the nucleolus have drawn special attention. According to Caspersson [2] the commencement of cell growth is followed by the appearance of nucleolar material inside the chromocenter. This is subsequently inflated and "exploded" by the accumulation of nucleolar substances. The chromocenter related to the nucleolus or nucleolus-associated chromatin is thus assumed to be a leading factor in the system for cytoplasmic protein formation [3]. In certain virus infections characterized by formation of intranuclear inclusions, the nucleolus-associated chromatin has been considered to be concerned with protein formation in order to fulfill needs of virus propagation [2, 7]. In spite of the considerable significance of these observations, morphological details of the structures still are incomplete, especially concerning the structural interrelation between the Feulgen-negative and positive components or between the nucleolus and the

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nucleolus-associated chromatin. The morphological specificity and the production site of the latter need to be clarified. With the advantage of improved techniques and the intention of contributing to the clarification of these problems, we have undertaken the present studies.

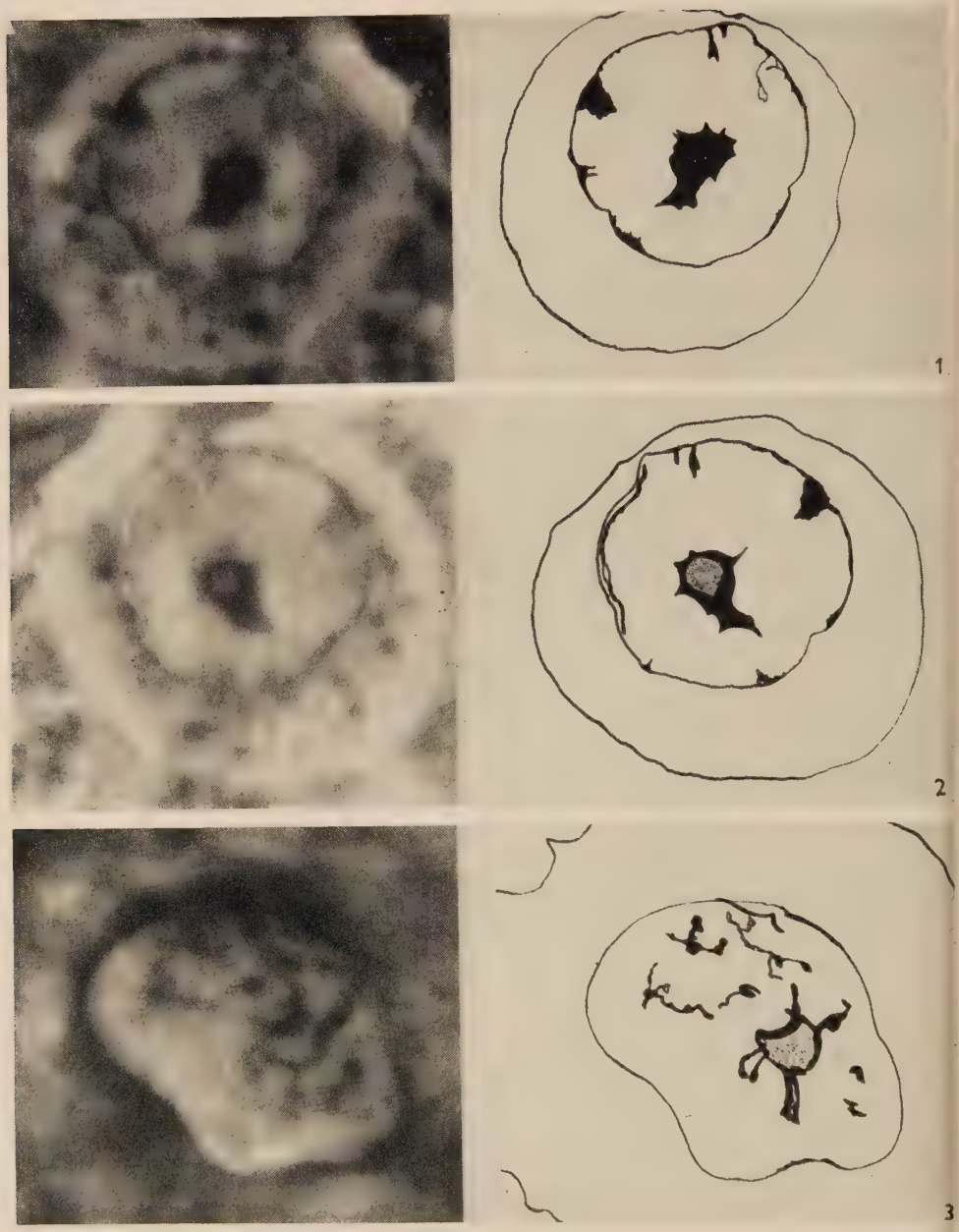
Rat lymphoblasts were chosen for this study because most of them have a single large nucleolus, stained as a well-defined body. The mesenteric lymphnodes were surgically removed from six normal Wayne-Pink-eye rats each weighing about 100 g. In each case small pieces of cortex were rinsed in an equal volume of horse serum. One or two drops of the resulting fluid, in which free lymphoid cells were suspended, were placed between a cover slip and a microscope slide. The preparation was observed by phase contrast microscopy.

The findings obtained from the six animals were similar. The lymphoblasts were easily identified. The nucleus in these cells was bordered by a distinct membrane. In the center of the nucleus a large dark body was observed which is generally regarded as the plasmosome (Fig. 1). This appeared uniformly dark and without organized structure. Gentle pressure was given to the cover slip to flatten the cell. A light area appeared in the center of the dark body (Fig. 2). When stronger pressure was given to the cover slip the light central area became larger, and the dark outer zone was loosened. Further pressure caused disintegration of the loosened structure of the outer zone disclosing one long and two equally medium-sized, dark, double-stranded threads, partly attached to the light central zone and partly radiating from it (Fig. 3).

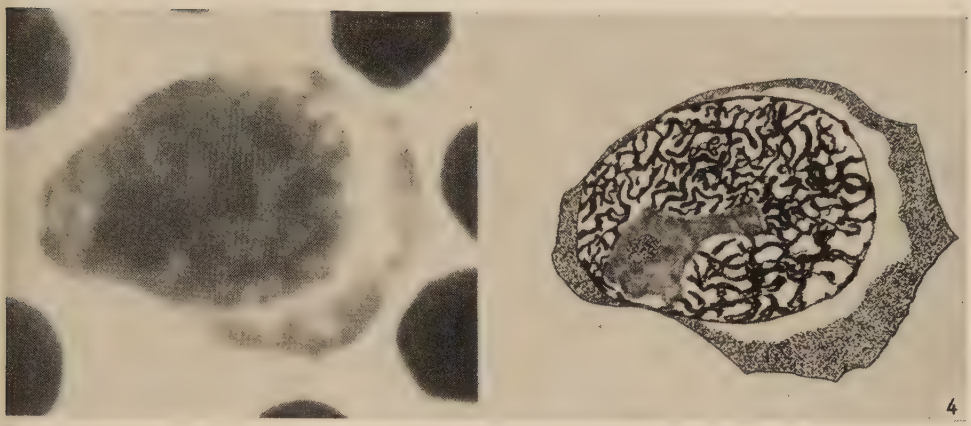
These observations suggest that the large dark body regarded as the plasmosome in the earlier preparations consists of two parts which were shown in the later preparations as the light central area and the dark outer zone composed of three dark threads. The central area very likely corresponds to the plasmosome itself, while the threads around it appear to be the nucleolus-associated chromatin.

Comparative observations were carried out using stained preparations. The suspension fluid of the lymphoid cells was obtained in a similar way as mentioned above. Film preparations made from the fluid were fixed with Carnoy solution and stained with Unna-Pappenheim stain. The Feulgen method was also used after treatment with 1 *N* HCl.

In the Unna-Pappenheim preparations the lymphoblast presented a large pyroninophilic body which is usually regarded as the plasmosome. Careful observation, however, showed that the body was not uniformly pyroninophilic but consisted of a pyroninophilic small central area and a somewhat



Figs. 1–3. Phase-contrast photomicrographs of the fresh lymphoblast from rat. Leitz-Wetzlar Periplan 10 × and Apo Oel 2 mm were used. The plasmosome is demonstrated as a uniformly dark body under the ordinary condition (Fig. 1). When the cell is flattened, a light zone appears in the center of the dark body (Fig. 2). When further compressed, the outer dark zone disintegrates into one long and two medium sized double-stranded threads radiating from the light zone (Fig. 3).



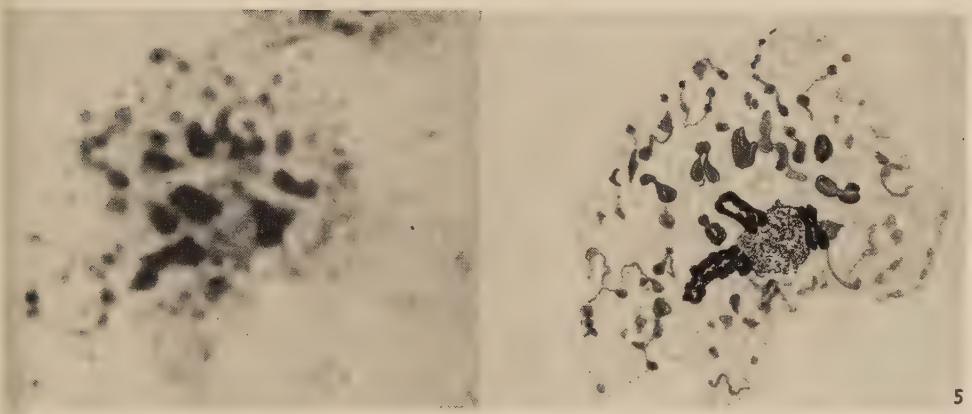


Fig. 4. A photomicrograph of the rat lymphoblast stained with Unna-Pappenheim stain. Leitz-Wetzlar Periplan 10 × and Apo Oel 2 mm were used. A purely pyroninophilic small inner zone and a somewhat amphophilic outer zone are vaguely distinguishable in the plasmosome.
Fig. 5. A photomicrograph of the swollen, flattened rat lymphoblast stained with Unna-Pappenheim stain. Leitz-Wetzlar Periplan 10 × and Apo Oel 2 mm were used. One long and two medium-sized double-stranded bodies are joined to a purely pyroninophilic body at their subterminal regions.

amphophilic outer zone (Fig. 4). By Feulgen stain, the former was negative while the latter was positive.

Stained preparations of the swollen, flattened lymphoblasts were obtained by pretreatment with a hypotonic solution. The suspension fluid of lymphoid cells, prepared in a manner similar to the above, was diluted with an equal volume of 0.05 per cent solution of Sodium Acetate aspirated in a blood-count pipette and incubated at 3°C for 20 minutes. A drop of the fluid from

the pipette was carefully spread on a clean microscope slide. Extra-thin film preparations were fixed with Carnoy solution and stained with Unna-Pappenheim stain. A duplicate preparation, after treatment with 1*N* HCl, was stained by the Feulgen technique.

The large body of the lymphoblast nucleus showed finer structures. The central and outer zones were clearly distinguishable. The central area was purely pyroninophilic but Feulgen negative. The outer zone consisted of one long and two equally medium-sized, double-stranded amphophilic, Feulgen positive threads which were attached to the pyroninophilic central area at their subterminal regions and otherwise arranged radially from the central area (Fig. 5). The central area appears to be the plasmosome itself or pure nucleolus. The three threads attached to it are apparently constant in size, form and the region of attachment to the nucleolus. They correspond to the heterochromatic regions of the chromosomes forming the nucleolus-associated chromatin.

We have recently found in the rat lymphoblast at metaphase three special chromosomes with the secondary constrictions at their subterminal regions which were one member of the second pair and both members of the 7th pair of autosomes [8]. These chromosomes and the threads found in the present study show a remarkable coincidence in number, relative size and regions where the secondary constrictions are present or where the nucleolus attaches. The three threads might be three special chromosomes. Further, the secondary constrictions on the chromosome could be similarly responsible for organizing the nucleolus, as the SAT-zone or nucleolus-organizer in plants and lower animals.

In conclusion, the nucleolus-associated chromatin of the rat lymphoblast is found to be composed of three heterochromatic threads attached to the nucleolus. These threads morphologically correspond to the special autosomes with the secondary constrictions which may be responsible for formation of the nucleolus.

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THE INTRACELLULAR DISTRIBUTION OF PROTEIN-BOUND AZO DYE IN RAT LIVER

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As has been shown by Miller and Miller [9] the carcinogenic action of azo dyes on rat liver is correlated with a binding of azo dye to liver cell proteins. It has often been suggested [9, 10, 17, 18], mainly on the basis of the important observations by the Miller group, that this binding process may in some way be engaged in the tumor induction proper, possibly by interfering with mechanisms of importance for the normal production of specific cell proteins.

Experiments with labeled amino acids, performed *in vivo* as well as *in vitro* [5, 2, 16, 6, 1, 8, 7], have shown that those structures of the cytoplasm which can be recovered from cell homogenates in the form of microsomes are the sites of a particularly active protein metabolism. The aim of the present investigation has been to determine whether there is a parallelism in this respect between the binding of azo dye and the amino acid incorporation.

The distribution of protein-bound azo dye between different fractions of rat liver cells has been studied previously [10, 11, 12, 13], but only on animals, which have been supplied continuously with the azo compound in the diet over a rather long period. In experiments of this type, in which the distribution of the dye between the cell constituents may fairly soon be determined by a combination of more or less interdependent steady state relationships, the highest content of azo dye-protein complex has constantly been observed in the soluble protein fraction [cf. 19]. It will be shown below that the early pattern of dye incorporation is different. During the early period following the administration of single doses of 3-methyl-4′-dimethylaminoazobenzene the highest content of protein-bound azo dye was found in the proteins of the microsome fractions.

EXPERIMENTAL

Albino rats weighing 175–200 g were used. Prior to the experiments they were maintained for 6–10 days on a low-protein basal diet, used previously for tumor production by Hoffman and Schechtman [4].

The 3-methyl-4′-dimethylaminoazobenzene (m-DAB) was synthesized from m-toluidine [3] and was administered to the rats in the form of a 2 per cent (weight) solution in olive oil. Of this solution, 2 ml were introduced into the stomach and 1 ml into the abdominal cavity of each rat. For each experiment 4 rats were treated simultaneously. The animals were then deprived of food and water for 5 hours, after which time they were again maintained on the basal diet. In one series of experiments the animals were transferred instead to a basal diet containing 0.06 per cent (weight) of m-DAB.

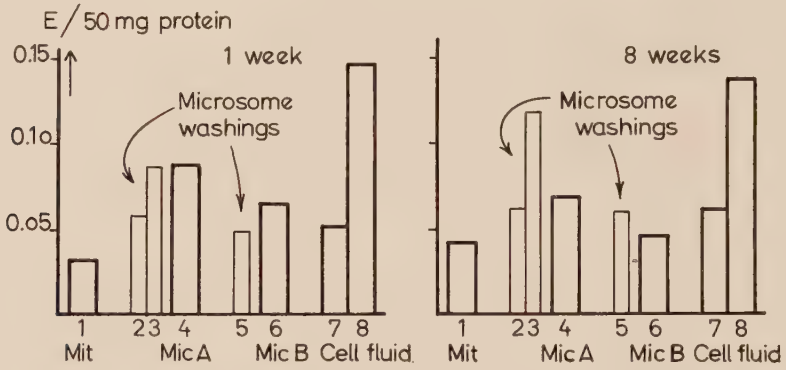


Fig. 1. Contents of protein-bound azo dye in liver cytoplasm fractions from rats, maintained for different lengths of time on a low-protein basal diet, containing 0.06 per cent 3-methyl-4′-dimethylaminoazobenzene. Each experiment includes 4 animals.

Bars 1: Mitochondrial fraction.
Bars 2: First washings of microsomes A: ultracentrifuge sediments.
Bars 3: „ „ „ „ „ „ supernates.
Bars 4: Microsomal fraction A.
Bars 5: Washings of microsomes B.
Bars 6: Microsomal fraction B.
Bars 7: Ca-precipitated cell-fluid proteins (soluble nucleoproteins).
Bars 8: Cell fluid proteins non-precipitable with $CaCl_2$.

The rats were killed by decapitation 3 hours to 12 days after the administration of the azo dye. Before being sacrificed, they were fasted for 12–15 hours. The livers were thoroughly perfused *in situ*, then carefully minced in an ice-cold solution of 0.25*M* sucrose and 0.005*M* versene. Following repeated washings, the suspensions were homogenized in a glass homogenizer. The pooled homogenates from the 4 animals included in each experiment were diluted with approximately 150 ml of sucrose-versene solution per 10 g of liver tissue.

The fractionations were performed in a Spinco preparative ultracentrifuge at 0–3°C. The homogenates were first centrifuged for 5 minutes at 500 g. The sediments were suspended by slight homogenization in approximately 100 ml of sucrose per 10 g of liver, and were centrifuged as before. The supernatant fluids from the two centrifugations were combined and were centrifuged again at the same speed. The sediments obtained at these centrifugations were discarded.

The cloudy supernatant fluids were centrifuged for 10 minutes at 5000 g. The sediments obtained were washed twice in sucrose by centrifugations for 10 minutes

at 5000 g. The washed sediments were mainly composed of mitochondria ("Mitochondrial fraction").

The cytoplasmic suspensions were further centrifuged for 10 minutes at 7000 g, the sediments being discarded. The supernatant fluids were centrifuged for 20 minutes at 20,000 g. Pink, glassy pellets were obtained. These sediments were washed twice in sucrose by centrifugations for 20 minutes at 20,000 g ("Microsomal fraction A"). The first washings were centrifuged for 60 minutes at 70,000 g. Microsome-like pellets were formed.

The cytoplasmic supernates were finally centrifuged for 60 minutes at 70,000 g. The pellets obtained were washed in sucrose by centrifugation for 40 minutes at 105,000 g ("Microsomal fraction B").

Dilute $CaCl_2$ was added to the final cytoplasmic supernates to a final concentration of 0.015*M*. The precipitated nucleoproteins [7, cf. 15] were centrifuged off and washed in water.

For the preparation of protein samples the fractions were treated according to a slightly modified Schneider [14] procedure. The proteins were finally dried with ethanol and ether. Samples containing 50 mg of dry protein powder were hydrolyzed with ethanol-KOH for 20 hours at 80°C and analyzed for azo dye as described by Miller and Miller [9]. As a rule, the Schneider extractions and azo dye determinations were run in duplicates.

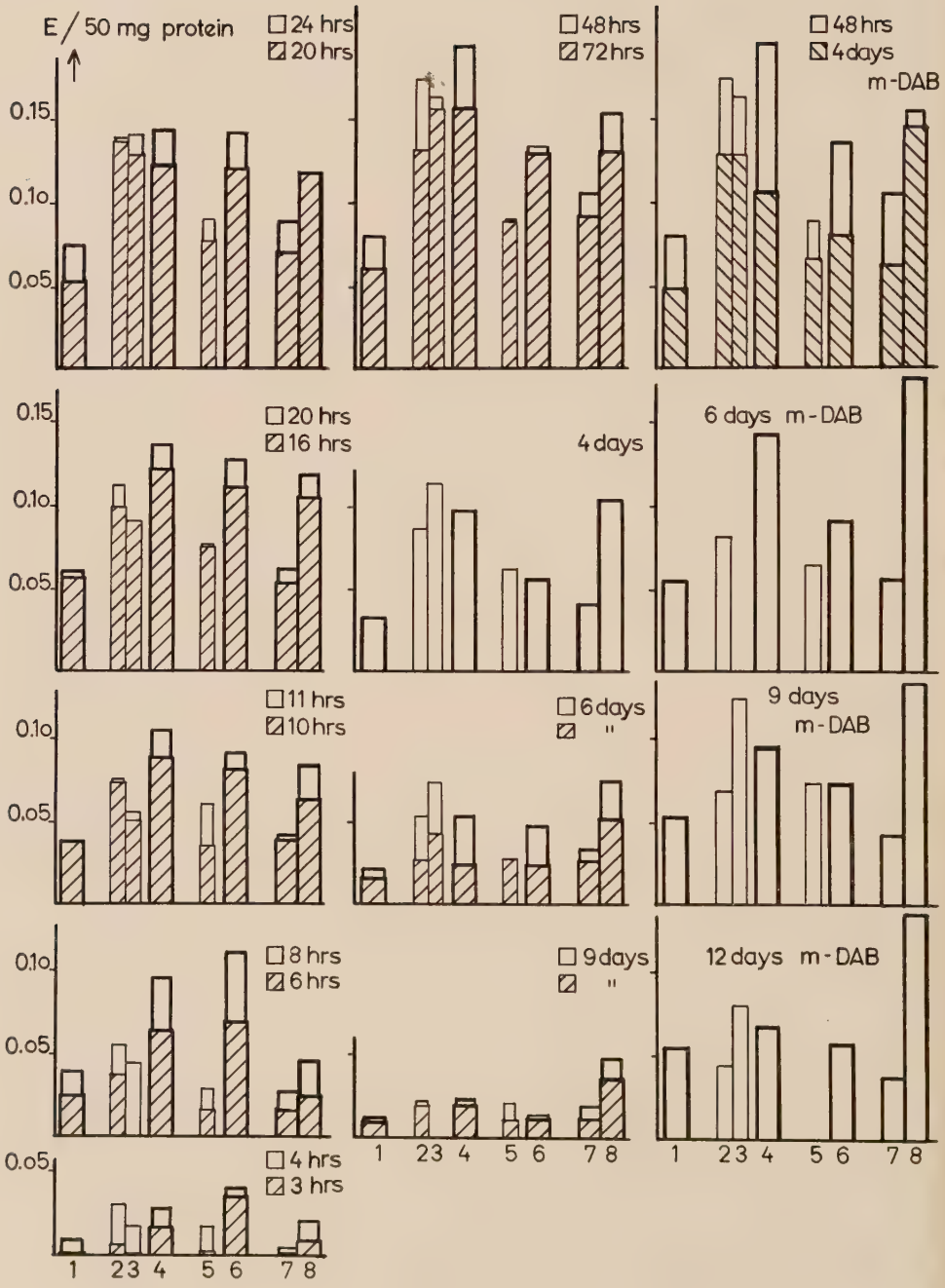
For comparison, some fractionation experiments were performed on livers from rats which had been maintained for different lengths of time on a basal diet containing 0.06 per cent (weight) m-DAB.

RESULTS

The deposits of 3-methyl-4′-dimethylaminoazobenzene (m-DAB), introduced into the rats, were largely exhausted after 50–60 hours, at which time the adipose tissue of the mesenteries had lost most of its yellow color. During this initial period, characterized by a continuous supply of m-DAB from the deposits, the contents of azo dye-protein complex steadily increased in all liver fractions (Fig. 2, left column).

The analytical methods which were used could not be satisfactorily applied to experiments of shorter duration than 3 hours. The experimental data available show, however, that during the whole period of rapid increase in color intensity, and particularly during the first 10 hours, the proteins of the microsome fractions had incorporated the dye more rapidly than the other fractions investigated. During the first 8 hours this fact was particularly obvious in the proteins of the small microsomes (Fig. 2, bars 6). Later, the large microsomes (bars 4) became superior in respect of dye content.

Towards the end of the initial period of rapid dye uptake the distribution of the protein-bound dye between the soluble and non-soluble protein fractions gradually changed. The content of bound dye in the soluble protein


E / 50 mg protein
24 hrs
20 hrs
48 hrs
72 hrs
48 hrs
4 days
m-DAB
0.15
0.10
0.05
20 hrs
16 hrs
4 days
6 days m-DAB
11 hrs
10 hrs
6 days
"
9 days
m-DAB
8 hrs
6 hrs
9 days
"
12 days m-DAB
4 hrs
3 hrs
1 2 3 4 5 6 7 8

fraction, obtained after $CaCl_2$ precipitation (Fig. 2, bars 8) successively increased in relation to the dye contents of the other fractions, and it even surpassed the dye content observed in the fraction of small microsomes (48 hours).

When the depots of azo dye had been emptied, the content of protein-bound dye in the liver gradually decreased (Fig. 2, middle column). The decrease was especially rapid in the particulate fractions, and after a few days the soluble proteins, non-precipitable with $CaCl_2$, had considerably higher dye content than the proteins of the particulate fractions. The pattern of distribution of protein-bound dye between the cell fractions had thus become very similar to the pattern (Fig. 1) observed in rats, continuously supplied with low concentrations of azo dye in the diet [11, 12, 13].

A similar distribution was obtained in the series of experiments, in which, 5 hours after the injections of m-DAB, the animals had been transferred to basal diet containing 0.06 per cent m-DAB (Fig. 2, right column).

In all experiments the mitochondrial fraction and the soluble nucleoproteins (Fig. 2, bars 1 and 7) showed lower contents of dye-protein complex than the fractions just described. The washings of the small microsomes (bars 5), which contain considerable amounts of nucleoprotein [7], showed approximately the same content of protein-bound dye as the soluble nucleoproteins in the cell fluid fraction (bars 7). (In a few experiments some protein material may have been released from the microsomes proper by the washing procedure, as indicated by a slightly increased dye content in the proteins of these washings.)

As was mentioned above, the washings of the large microsome fractions were divided by centrifugation into a microsome-like pellet and a clear supernatant fluid. As might be anticipated from the data already mentioned, the proteins of the pellets (bars 2) during the initial period (3–48 hours) showed higher dye contents than the proteins of the supernates (bars 3), while the opposite was true later, during the period of regression.

DISCUSSION

Under the experimental conditions described the microsome material of rat liver was characterized by a particularly rapid initial rate of formation

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Fig. 2. Contents of protein-bound azo dye in fractions of rat liver cytoplasm. After the administration of a single dose of m-DAB by injection, the rats were maintained for different lengths of time on low-protein basal diet, or on basal diet containing 0.06 per cent m-DAB. Each experiment includes 4 animals. The fractions are symbolized in the same way as in Fig. 1.

of azo dye-protein complexes. It seems justified, therefore, to propose a major role of the microsome structures in the process of incorporation of carcinogenic azo dyes into liver cell proteins.

In view of the importance of the microsome structures in protein metabolism [5, 2, 16, 6, 1, 8, 7], it is tempting to speculate on these structures as conceivable effective centers during the early stages of induction of azo dye tumors. Possibly, certain kinds of azo-proteins, formed in the microsome structures are secondarily transferred to the cell fluid.

It is our intention to study the incorporation process at earlier stages, i.e. during the first few minutes after the administration of azo dye, by means of isotope technique. As an additional advantage, it will be possible in that way to avoid the undesirably high dosages of azo dye, which had to be used in the present experiments.

SUMMARY

Single doses of 3-methyl-4′-dimethylaminoazobenzene were given to rats by injection. The distribution of protein-bound azo dye between different fractions of the liver cytoplasm was investigated at different times after the injections. During the early period of rapid uptake of the dye, the proteins of the microsome fractions had a considerably higher content of bound dye than the proteins of the other cytoplasm fractions. Later, during the period of regression, a soluble protein fraction, non-precipitable with $CaCl_2$, showed the highest dye content.

The experiments suggest that 3-methyl-4′-dimethylaminoazobenzene, under the experimental conditions described, becomes associated, at a particularly rapid rate, with those cytoplasm structures which, upon isolation, are concentrated in the microsome fractions.

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THE BEHAVIOUR OF NUCLEOLI IN *PHAEDRANASSA CARMIOLI*

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Since the first mention of the nucleolus, which was probably made by Fontana in 1781 [8], many studies have been centred around attempts to understand its formation and functions and also to analyse its composition. Much of this work has been usefully summarised by Gates [10], Vanderlyn [30] and Hughes [15]. This paper illustrates differences between nucleoli within pollen mother cells of *Phaedranassa carmioli (Amaryllidaceae)* and indicates that these differences may inhibit true nucleolar fusion during the prophase of meiosis.

EXPERIMENTAL PROCEDURE

Anthers, young developing ovules and root-tips were each fixed in a variety of fixatives (e.g., Acetic alcohol (1:3), Carnoy, La Cour's 2 B.D. and Lewitsky). Some of the material was embedded in wax and sectioned at 6 or 8 μ, the rest being used in the preparation of squashes. The stains employed are given in a later section of this paper (Table I).

OBSERVATIONS

Meiotic nucleoli

Pollen mother cells of *Phaedranassa carmioli* (which normally contain 23 bivalents at M I) were examined after staining with aceto-carmine. During the prophase of meiosis a large nucleolus is seen, apparently organised by the ends of one of the pairs of long chromosomes. This nucleolus, instead of being homogeneous, is made up of two distinct regions one being more heavily stained than the other. The staining capacity of this region, with aceto-carmine, markedly increases when the acetic-alcohol contains a trace of ferric chloride (Fig. 1).

The inner, and more densely stained, zone of the nucleolus is apparently spherical, totally enclosed by the outer zone and always at its periphery in

TABLE I

Staining reactions of the inner and outer zones of the nucleolus.

Fixative	Staining method	Reference	Colour of inner zone	Colour of outer zone
Acetic alcohol	Carmine	cf. [5]	Reddish	Pale red
Acetic alcohol plus iron	Carmine		Reddish brown	Red
Aceto-orcein	Aceto-orcein		Red	Pale red
Carnoy	Methyl Green Pyronin		Brown-red	Red
2 B.D.	Crystal Violet		Blue	Blue
Carnoy	Feulgen	[7]	Weak Feulgen reaction	Clear
Lewitsky	Feulgen, Light Green and Orange G.	—	Orange	Green (+orange?)
Lewitsky	Feulgen and Light Green	[28]	Weak Feulgen reaction	Green
Carnoy	Methyl Green	[17]	Pale green	Clear

association with the nucleolar-organising chromosomes of the complement (Fig. 1). The two zones persist from early meiotic prophase until the nucleolus disperses just before metaphase. The only visible change is an apparent decrease in volume of the inner zone as diakinesis is approached.

It thus seems possible that the nucleolus might consist of two separate zones differing quantitatively and/or qualitatively in chemical state. In order to test these possibilities the staining methods given in Table I were used.

The method used for Feulgen, Light Green and Orange G was similar to that of Semmens and Bhaduri [28] but the 95 per cent alcohol was saturated with Orange G as well as Light Green.

The inner zone of the nucleolus is faintly stained with Feulgen, Methyl Green and Orcein and this could indicate the presence of deoxyribose nucleic acid. To test this possibility sections were treated with deoxyribonuclease, after the method of Jacobson and Webb [16], and then stained with Feulgen. There was no staining of the chromosomes but the nucleolus remained weakly Feulgen-positive. This then suggests that the staining is probably related to the presence of free aldehydes. Gomori suggests that in tissues treated with lipid solvents aldehyde reactions under specific conditions are attributed to carbohydrates [11].

The following cytochemical tests were carried out for:

Free aldehydes.—Following the reaction given by Gomori [11] the whole nucleolus is faintly stained showing the presence of some free aldehyde but there is no zonal differentiation.

Polysaccharides.—Using an alkaline solution of silver nitrate, as given by Gomori [11], the inner zone is shown to contain some polysaccharide. The outer zone, however, does not stain.

Following the Hotchkiss method [14] the whole nucleolus stains, indicating the presence of water-soluble polysaccharide. There is no difference between the two zones.

Arginine.—After the Sakaguchi arginine reaction [26] the inner zone of the nucleolus stains more deeply than the outer zone (Fig. 4). This may indicate a greater amount of arginine but because of the smallness of the inner zone there is the possibility that it might be more highly concentrated there.

Phospholipids.—A modification by L. F. La Cour and J. Chayen of J. R. Baker's acid haematin reaction for phospholipids [1] was used. The inner zone of the nucleolus gives an intense blue-black reaction indicating phospholipid to be present in this region only (Fig. 5). The possibility that this reaction may not be sufficiently sensitive for small amounts of phospholipid cannot be disregarded, however.

In many of the preparations (e.g., Fig. 2) a clear region is visible between the nucleolar zones. This suggests atypical fusion of two nucleoli due apparently to a difference in chemical state as reflected by the differential staining.

Confirmation of this supposedly heterogeneous condition is provided by an experiment designed to separate the nucleoli by creating failure of meiotic chromosome pairing. This was attained by keeping a plant at 40°C for 24 hours. When meiotic prophase is examined immediately after the heat shock some failure of chromosome pairing is at once obvious (Fig. 7). In many of these nuclei a large single nucleolus is replaced by two medium-sized nucleoli. In addition these nucleoli are still differentially stainable with aceto-carmine (Fig. 6). When other stains and cytochemical reactions were used on this heat-treated material, however, no differential staining could be obtained. This applied both to the "fused" and the separated nucleoli. The

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Pollen mother cells of *Phaedranassa carmioli*.

Fig. 1. Diakinesis. Fixation acetic alcohol (1 : 3) plus ferric chloride solution. Squashed and stained in aceto-carmine. × 1400.

Fig. 2. Pachytene. Smeared and fixed in acetic alcohol (1 : 3). Stained in Methyl Green pyronin. Note clear crescent in nucleolus. × 2100.

Fig. 3. Diplotene. Fixation Lewitsky. Sectioned at 8μ and stained in Feulgen, Light Green and Orange G. × 2100.

Fig. 4. Pachytene. Fixation Lewitsky. Sectioned at 8μ and tested for arginine. × 2100.

Fig. 5. Leptotene. Fixation Lewitsky. Sectioned at 8μ. Acid haematin reaction for phospholipids as modified by L. F. La Cour and J. Chayen. × 2100.

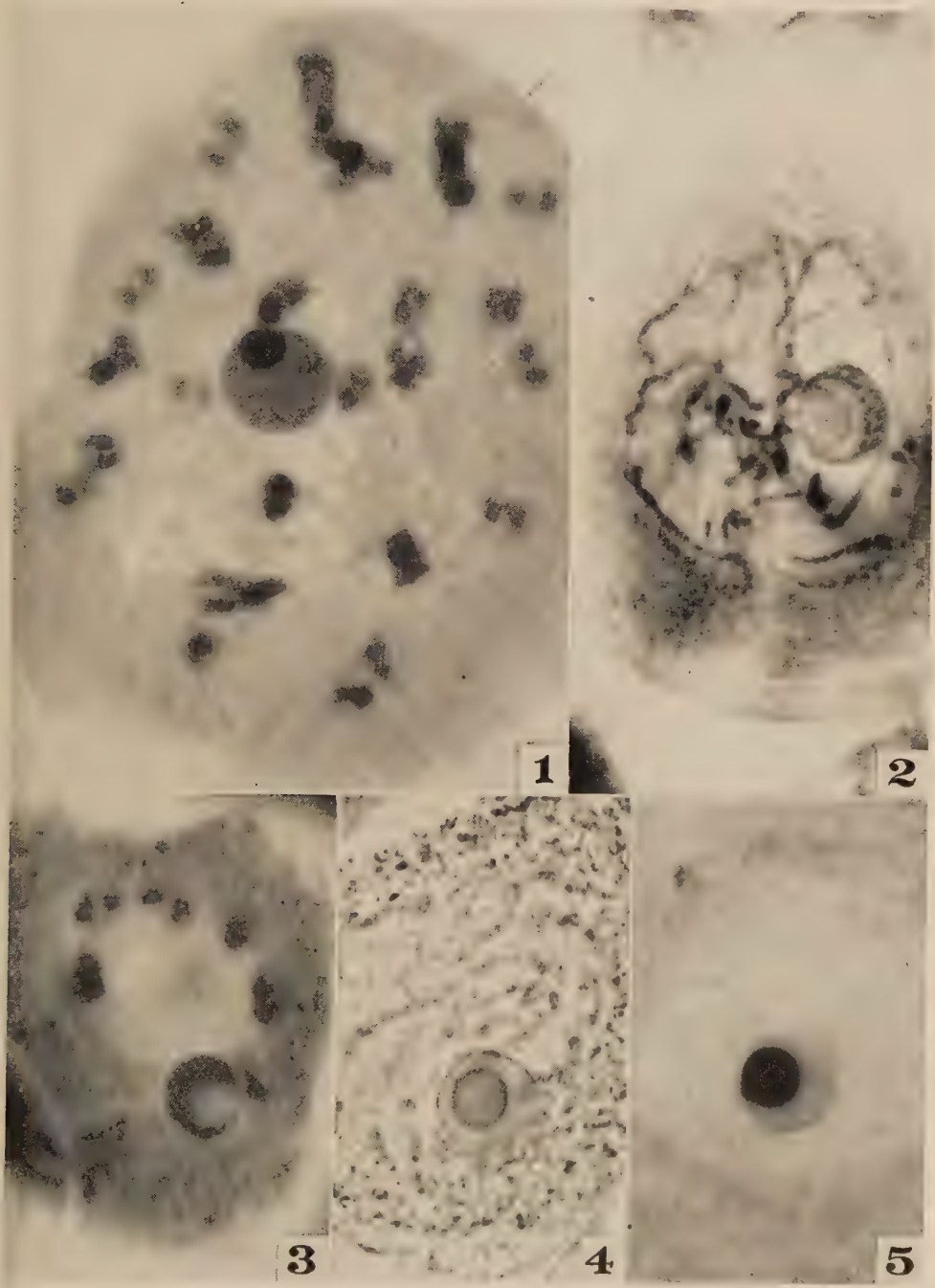
1
2
3
4
5

TABLE II

Colour reactions of the two nucleoli in normal and heat-treated meiotic cells.

Cytochemical test or staining method	Normal		Heat-treated[1]	
	Inner nucleolus	Outer nucleolus	Inner nucleolus	Outer nucleolus
Phospholipid	Black	Clear	Black	Black
Arginine	Orange	Pale orange	Orange	Orange
Polysaccharide (alkaline $AgNO_3$)	Orange	Clear	Orange	Orange
Feulgen, Light Green and Orange G.	Orange	Green	Orange	Orange
Methyl Green Pyronin	Brown-red	Red	?Brown-red?	Red
Carmine + Iron	Red-brown	Red	Red-brown	Red

[1] The colour reaction of the two nucleoli is the same whether they are "fused" or separate.

only possible exception was with methyl green-pyronin when the two nucleoli appeared to differ slightly in intensity of staining (Table II).

In some of these heat-treated nuclei, both before and during pachytene, there are three nucleoli—one large and two small—lying close together. This indicates the presence of more than two nucleolar organisers and is perhaps related to premature chromosome splitting following the heat shock. The separate nature of the nucleoli is also an indication that all nucleolar fusion does not take place, or is not complete, until the beginning of meiotic prophase in *Phaedranassa*. This has also been demonstrated in *Tradescantia bracteata* [29].

Another peculiarity common to many of the nuclei is the organisation of several smaller and less densely-staining nucleoli by the ends of some of the other chromosomes of the complement (Fig. 7). This condition is not seen in untreated material.

Mitotic nucleoli

Apart from pollen mother cells other tissues were also examined—root-tips, nucellus, endosperm, etc. Endosperm is particularly suitable for the study of nucleoli because an increase in the size and ploidy of the nuclei leads to the formation of larger nucleoli. In all these tissues, whether there

Fig. 6. Zygotene. Prepared as Fig. 1. Immediately after the 24-hour heat treatment. Note the two differentially stained nucleoli. × 1400.
Fig. 7. Pachytene. Prepared as Fig. 1. Immediately after the 24-hour heat treatment. Note the failure of chromosome pairing—particularly at the chromosome ends—and the several small, weakly-stained nucleoli. × 1400.

6

7

was nucleolar fusion or not, no differential reactivity could be obtained by any of the staining methods or cytochemical reactions. In all cases the nucleoli appeared homogeneous, apart from the normal occurrence of vacuoles.

DISCUSSION

The fusion of nucleoli in both mitotic and meiotic nuclei is common in plants and seems to involve a blending or coalescence of the nucleoar protein content within an enlarged "envelope" consisting of ribonucleoprotein probably together with phospholipid.

It is generally agreed that nucleoli are formed at telophase and that fusion can follow this organisation. That nucleoli are formed at specific regions of the chromosomes is also generally accepted [e.g., 13 and 19]. It has been reported, however, that droplets or layers of pronucleolar material can be formed at telophase by the "periplasm" (a mixture of cytoplasm and nucleoplasm). These droplets later coalesce and accumulate at the nucleolar zones of the chromosomes [24]. The fact that nucleoli may differ chemically makes this theory improbable. It seems far more likely that nucleoli are organised by and at specific regions of chromosomes.

In *Phaedranassa*, at meiosis, there is apparently no blending or coalescence of the two nucleoli but merely the inclusion of one nucleolus within the other. Whether this takes the form of one sphere enclosed by another or whether the outer nucleolus becomes invaginated to accommodate the other it is impossible to say. It is also possible that the coacervate nature of the nucleolar contents is inhibited by chemical differences while the membranes behave quite normally. Thus a state of equilibrium is achieved but this is not stable as is evidenced by the gradual diminution in volume of the included nucleolus as meiotic prophase advances.

One very striking qualitative difference exists between the two nucleoli. There seems to be a complete absence of phospholipid in the outer one but a high concentration of it in the inner one. Another possible variation is that of arginine, indicating differences in protein content. As the phospholipid is probably protein-linked (La Cour and Chayen, unpub.) and as an acetic alcohol fixative would remove much of this complex, the differential staining with aceto-carmine may be due to some other compound not yet determined. The reactions of the two nucleoli to the various mixtures of stains also indicate other chemical differences.

After the heat shock, however, many of these differences no longer exist or cannot be demonstrated. It is reasonable to assume that changes in

temperature will change the rates of synthesis and breakdown of products in the cell. As a result both the nucleoli stain and react in almost the same way, although some difference can still be seen after staining with aceto-carmine. This differential staining may be associated with ribose nucleic acid as demonstrated by methyl green-pyronin (Table II).

A nucleolus apparently similar in composition to that of *Phaedranassa* is also present in the pollen mother cells of *Hymenocallis*. On examining pollen mother cells of *Hippeastrum*, *Sprekelia* and *Urceolina*, however, the nucleoli are found to be quite normal. The atypical nucleolar behaviour cannot, therefore, be characteristic of the *Amaryllidaceae*.

McGill [20] reported that the nucleoli during oogenesis in the dragon fly were double, there being a basiphyllic portion surrounding an oxyphilic one. Another example of a nucleolus composed of two regions is given by Gates [9]. Nucleoli of fresh eggs of *Asterias*, *Mactra* and *Chaetopterus* were shown to be of "two parts like immiscible fluids, one enclosed within the other. The outer portion is more quickly soluble in tap water".

The process of nucleolar "budding" can be compared with nucleolar fusion. It has been reported in many animals [10], in rice [27, 23], *Populus* [6] and *Antirrhinum* [25]. It has also been discussed by Mensinkai [22] who put forward an alternative explanation of his observations. He stated that "it is merely a stage in the progressive fusion of homologous" nucleoli. This can be further expanded to include "non-homologous nucleoli [e.g., 2 and 29]". The fusion of these nucleoli, like that of heterochromatin in the interphase nuclei of some plants [4], will depend upon the position of the organisers or the chromosomes. For example, in *Pisum* nucleoli are organised at or near the centromeres and the chances of their fusion are greatly enhanced at telophase [12].

It is of particular interest that in *Phaedranassa* only the nucleoli seen at meiosis show a heterogeneous condition. Presumably there is a genic difference between the nucleolar organisers which only becomes apparent at different levels of metabolic activity of the cell. Mitotic and meiotic cells will certainly differ in this respect. This interpretation is supported by the effect of high temperature in restoring an almost typical condition as a result of alterations in cell metabolism.

After the heat shock "secondary" nucleoli were organized by normally quiescent organisers in *Phaedranassa*. Similar reports have been given by Heitz [13] and McClintock [19] and also by Matsuura [21] when chromosomes have been isolated in micronuclei. Plants with terminally organised nucleoli seem to have an irregular type of nucleolar organisation. For

example, in *Trillium* [3] and in some species of *Fritillaria* [18] there are no constant nucleolar organisers and the nucleoli can be formed by the ends of any of the chromosomes.

Thus in *Phaedranassa* the regularly appearing nucleoli differ qualitatively according to gene activity, as is reflected by their atypical fusion at meiosis and normal fusion at mitosis. In addition, normally inactive nucleolar organisers are apparently stimulated into activity by abnormal environmental conditions.

SUMMARY

1. The nucleoli of pollen mother cells of *Phaedranassa carmioli* are shown to contain, after several different staining methods, a dense inner region which is always associated with the nucleolar-organising chromosomes of the complement. No such region is visible in the nucleoli of somatic cells.

2. Differences are apparent between these nucleolar zones after the phospholipid, arginine and aldehyde reactions, and also after the use of various combinations of stains.

3. A 24-hour heat shock induces failure of chromosome pairing at meiosis. Two separate, but still differentially carmine-stained, nucleoli are then seen. However, most of the differences between the nucleoli, whether they are "fused" or separate, are no longer detectable. This is presumably due to the high temperature altering the cell metabolism.

4. Diverging chemical activity of the nucleoli inhibits normal nucleolar fusion during meiosis. A state of equilibrium results. Since nucleoli in somatic cells behave typically this unusual condition is presumably related to differences in gene expression at mitosis and meiosis.

5. Further interesting results followed the heat shock. Normally quiescent terminal nucleolar organisers gave rise to small, very weakly-stained nucleoli. This instability of nucleolar organisation is characteristic of several plants with terminally organised nucleoli.

I wish to thank Mr. L. F. La Cour for his helpful criticism and advice.

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THE EFFECT OF GLUCOSAMINE ON HUMAN EPIDERMOID CARCINOMA CELLS IN TISSUE CULTURE

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The growth inhibiting activity of D-glucosamine for tumour cells has recently attracted the attention of a number of workers, but contradictory results have been reported.

Quastel and Cantero [11] have claimed that aqueous neutral solutions of D-glucosamine hydrochloride, when injected daily intraperitoneally into tumour bearing mice (sarcoma 37), produce marked growth inhibiting and cytotoxic effects on the tumour. Lettré [10] was unable to confirm these results. He could observe no growth inhibition by glucosamine on Ehrlich's mouse ascites tumours and other mouse ascites tumours. Rubin *et al.* [12] described recently the growth inhibitory effect of D-glucosamine hydrochloride, mixtures of D-glucose and ammonium chloride, and D-glucose on cells in tissue culture. Woodward and Hudson [14] demonstrated the inhibitory action of D-glucosamine on the anaerobic fermentation and growth of yeast. The aerobic fermentation and respiration of these cells was only to a small extent affected. The action of D-glucosamine hydrochloride on chicken heart cells in tissue culture was studied by Ely *et al.* [5]. They found a reduction of the mitotic index and depression of growth. Astrup, Fischer, and Oehlenschlaeger [1] have shown that glucosamine cannot be a nutritional substitute for glucose in tissue culture media. It is also of interest to note the observations by Lea, Rhodes, and Borrell [9], showing the instability of glucosamine in the dry state in the presence of casein.

We considered it of interest to investigate the effect of D-glucosamine on human tumour cells, since D-glucosamine is a structural unit of a cytotoxic polysaccharide isolated from cultures of *Mycobacterium tuberculosis*. We found that the polysaccharide [15] and D-glucosamine were toxic and growth inhibiting for human carcinoma cells in tissue culture.[1]

Our present report is concerned with the toxic effect of D-glucosamine

[1] The authors wish to thank Rita Hansen and Ulla Bjørn Rasmussen for technical assistance.

hydrochloride on human tumour cells in tissue culture, and a study of the physico-chemical changes in the glucosamine molecule occurring under various conditions, including those prevailing during cultivation.

MATERIALS

The human epidermoid carcinoma cells Af (H. Ep. No. 2) [7, 8, 13] were chosen for the study. The cell strain is hardy, and has a uniform growth rate, increasing approximately three-fold in a week under the experimental conditions used. The strain has been growing in tissue culture for 2½ years. Gey's medium[1] and Morgan, Morton, and Parker's synthetic (199) medium were used as the fluid phase.

Most experiments were performed with D-glucosamine hydrochloride (Hoffman-La Roche, Basel). Another preparation of this compound was obtained from Prof. Hans Lettré, Heidelberg University, with which he had been unable to confirm the results of Quastel and Cantero. A third preparation was obtained from Dr. M. Abdel Akker, Cairo University. All three preparations gave essentially the same results.

METHODS

A modification of a tissue culture technique developed by Ebeling [4] was devised for studying growth and estimating the growth index. The image of the culture was traced when projected on paper at the beginning and end of the experiment. The area was subsequently measured with a planimeter. The characteristic sharp edge of the cultures was an advantage when measuring the projected image for growth estimation. The ease with which cultures were removed from the clot for transfer was another advantage.

Stock cultures from a single mother culture were grown in roller tubes until they were about 1 cm^2 in size. They were then divided diagonally into four equal parts, which were placed in four Porter flasks, one of the flasks serving as the control, and the remaining three as experiments. Each Porter flask contained three cultures, one of which was placed on a cover slip for staining. The clot on the bottom of the flask in which the cultures were embedded was thin, consisting of five drops of chick plasma and two drops of 1–1 chick embryo extract. The flasks were rotated in a roller drum for five days. Tissue culture area increase was determined by subtracting the original total tissue culture area per flask from the final total tissue culture area per flask. Subsequently the growth index was calculated, which was the figure obtained when the area increase (growth) per flask of the experimental cultures was divided by the area increase per flask of the control cultures. A growth index of more than one indicated stimulation, and less than one, depression of growth. The normal growth range had previously been determined. Each individual experiment represented four flasks, or 12 cultures.

Ultraviolet absorption spectra were measured with the Beckman spectrophotometer (model DU).

[1] Three parts Gey's balanced salt solution, 1 part penicillin (250 units per ml), 1 part streptomycin (250 microgram per ml), 2 parts 1–1 chick embryo extract, 1 part human serum, 1 part human placental serum, 1 part horse serum.

Determination of normal variation in control cultures.—When normal untreated cultures were measured in ten groups of five, in order to establish the normal variation, the growth index values ranged from .80 to 1.21. Ten averages for groups of ten ranged from .85 to 1.15. In addition a normal (control/control) value was established for each experiment. These values were 1.15, 1.09, and 1.05 for the three sets of experiments reported below, all within the normal range.

RESULTS

Inhibition studies.—Preliminary experiments using approximately 1000 cultures indicated that more than 1 mg/ml glucosamine in Gey's medium and medium 199 produced death of cells within 24 hours. Depressed growth could be detected from 1 mg to .001 micrograms per ml. It was noticed, however, that the same amounts of glucosamine did not always produce the same degree of cell damage and growth inhibition. Using medium 199 and a washed suspension of cells settled on cover slips, it could be shown that cell damage occurred within 8 hours, and that the presence of additional amounts of glucose did not effect the results. Since the pH of the medium containing large amounts of glucosamine sometimes became as acid as 6.5, controls were adjusted to this pH so that controls and experiments had the same pH.

Table I and Figs. 1–3 summarize the results of 3 experiments with glucosamine and glucose, confirming the preliminary work. Glucose was included in this study as the natural isoster to glucosamine.

TABLE I

Growth index values showing percentage of normal growth rate demonstrated by glucose and D-glucosamine treated human carcinoma tissue cultures.

No.	Medium	Reagent	Normal growth range	Control growth index	Mg reagent per ml										
					.000005	.00005	.0005	.005	.05	.5	1	5	10	50	500
					Growth index values										
1	G	g	.80–1.21	1.09							1.03	.86	.86	.60	
	G	ga	.80–1.21	1.09							.44	.12	.02	.00	
	199	ga	.80 1.21	1.05							.19	.05	.00	.00	
2	G	g	.80–1.21	1.09				1.05	1.06	.85		(.86)		(.60)	.00
	G	ga	.80–1.21	1.09	.72	1.04	.76	.41	.81	.58		(.12)		(.00)	.00
	199	ga	.80–1.21	1.05	.80	.54	.91	1.17	.73	.39		(.05)		(.00)	.00
3	G	g	.85–1.15	1.15				1.06	.92	.96		.90		.87	.00
	G	ga	.85–1.15	1.15	.76	1.06	.78	.60	.84	.53		.17		.00	.00

G = Gey's media; 199 = medium 199; g = glucose; ga = D-glucosamine.

Fig. 1 shows the effect of large amounts of D-glucosamine (1–50 mg/ml), using Gey's medium and medium 199. Glucosamine produced growth inhibition and dead cells in all dilutions. Glucose was added to 20 flasks of cultures in various amounts. 50 mg of glucose (in addition to the 2 mg/ml present in Gey's medium) appeared to

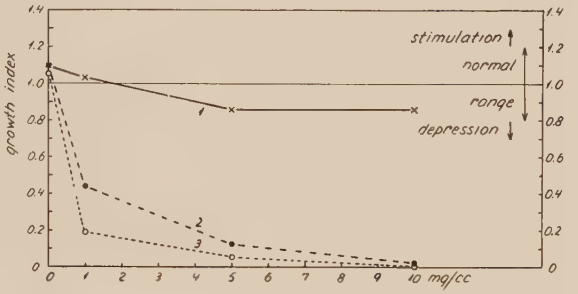


Fig. 1. Experiment 1 showing measurements of growth rates of human epidermoid carcinoma tissue cultures treated with (1) glucose in Gey's medium, (2) D-glucosamine in Gey's medium, (3) D-glucosamine in synthetic 199 medium.

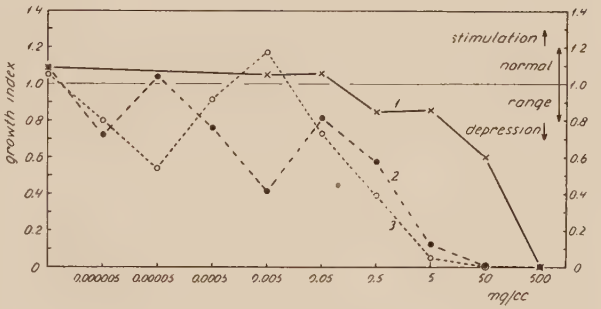


Fig. 2. Experiment 2 showing total range of measurements of growth rates of human epidermoid carcinoma tissue cultures treated with (1) glucose in Gey's medium, (2) D-glucosamine in Gey's medium, (3) D-glucosamine in synthetic 199 medium.

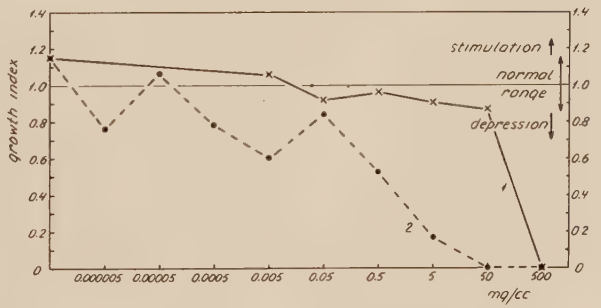


Fig. 3. Experiment 3 showing total range of measurements of growth rates of human epidermoid carcinoma tissue cultures treated with (1) glucose in Gey's medium, (2) D-glucosamine in Gey's medium.

be growth depressing, but additional amounts of 10, 5 and 1 mg/ml gave values within the normal growth range, .80 to 1.21. Untreated cultures showed a growth index of 1.09 in Gey's medium, and 1.05 in medium 199.

Fig. 2 shows the total range of effect of glucosamine in Gey's medium and medium 199. Cultures in Gey's medium were depressed by amounts of 500, 50, 5, and .5 mg/ml.

A peak of low normal growth was reached at .05 mg/ml, followed by depressed growth at 5 and .5 micrograms/ml. Normal growth values were reached at .05 micro-

grams, and slightly depressed growth was seen at .005 micrograms. In medium 199 cultures were disintegrated at 500 mg/ml. Depressed growth was demonstrated at .5 and .05 mg/ml, and normal growth was seen at 5 and .5 micrograms, with a depression at .05 micrograms, and a low normal value at .005 micrograms. 500 mg/ml glucose, in addition to the 2 mg/ml present in Gey's balanced salt solution, produced death of cultures in Gey's medium. 50 mg/ml appeared to be depressing, and all other concentrations from 0 to 5 mg/ml (in addition to the amount in the salt solution) were within the normal range (.80 to 1.21) established for the number of cultures used in the experiment.

Fig. 3 shows the results of the third study employing a total of 642 cultures. Each value represents ten individual experiments. Normal growth was not demonstrated in the presence of glucosamine, excepting at .05 micrograms/ml. A peak occurred at .05 mg/ml, and a depression at .005 micrograms followed normal growth at .05 micrograms/ml. Untreated control cultures showed a growth index of 1.15. The glucose results were consistent with the studies shown in Figs. 1 and 2. 500 mg/ml glucose in addition to the amount present in Gey's medium produced cell death. All other values from 50 mg/ml to zero mg/ml were within normal range, .85 to 1.15.

Cytological observations.—Cytological damage appeared first at the periphery of the cultures, where new actively growing cells appeared. Daily examination of the living cultures showed nuclear changes and granulation of the cytoplasm. Dividing cells and cells at the growing edge seemed more sensitive than resting cells. Dormant cultures [7], which had not received medium renewal or transfer for a period of several months, were not damaged by concentrations which were toxic for active cells. Staining techniques indicated that during initial damage, the cytoplasm became increasingly basophilic, and the nucleus increasingly acidophilic. At the end of the experiments, after methyl alcohol fixation, Mai-Grunwald-Giemsa stains were used routinely, and other stains were performed for special purposes.

Ultraviolet absorption spectra of aqueous solutions of D-*glucosamine.*—Water clear aqueous solutions containing 5 mg/ml of D-glucosamine hydrochloride were prepared (pH 5.8). Solutions at pH 5.5, 6.6, 7.0, 8.0, and 13 were also prepared.

Figs. 4–8 represent the ultraviolet absorption spectra of these glucosamine solutions taken at different time intervals.

Fig. 4 demonstrates that fresh solutions of D-glucosamine at pH 5.8 have no appreciable absorption in the ultraviolet. Figs. 5, 6, and 7 show the appearance of an absorption maximum at 272 mμ.

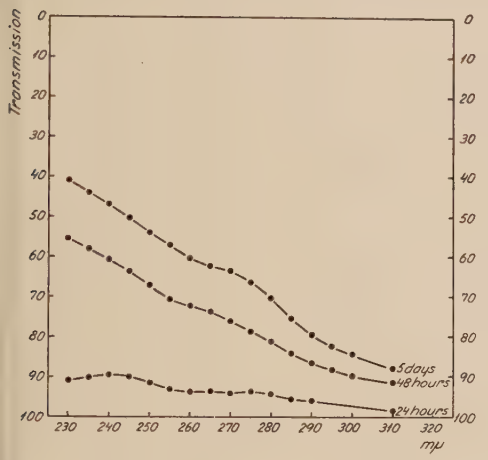
At pH 13 (Fig. 8) an absorption maximum at 275 mμ develops (probably caused by a dimerized pyrazine derivative).

These experiments show that aqueous solutions of D-glucosamine, especially at an alkaline pH, are unstable, giving rise to unidentified compounds with an absorption in ultraviolet.

The following figures were obtained when 0.5 per cent D-glucosamine in isotonic

solutions of NaCl, KCl, and Locke-Ringer (first adjusted to pH 7.0, becoming 6.6 on addition of glucosamine) were measured in the ultraviolet.

This demonstrates the instability of glucosamine in the presence of simple cations and anions; and thus emphasizes that the changes observed in more complex media, such as Gey's medium or medium 199 (see below) can occur under simple conditions.



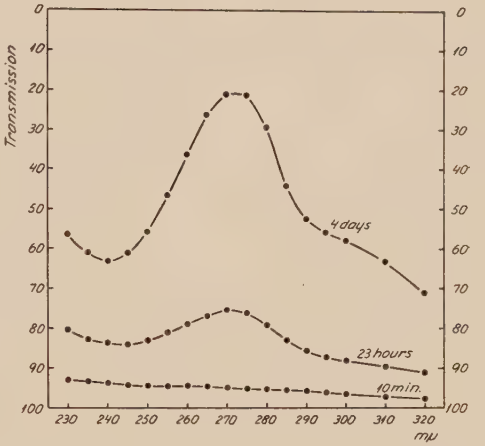
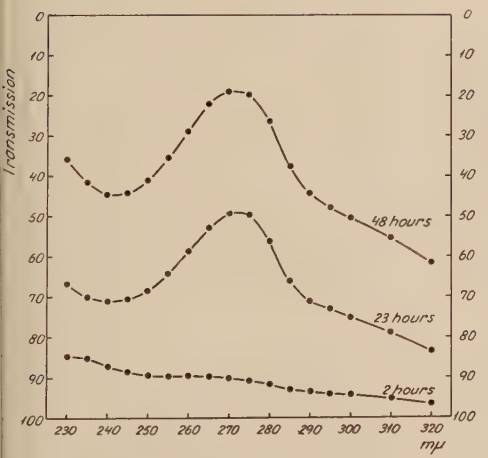


Fig. 4 (left). Ultraviolet absorption spectra of D-glucosamine-hydrochloride solutions at pH 5.8 and 37°C measured at different time intervals.

Fig. 5 (right). Ultraviolet absorption spectra of D-glucosamine-hydrochloride solutions at pH 6.6 and 37°C measured at different time intervals.



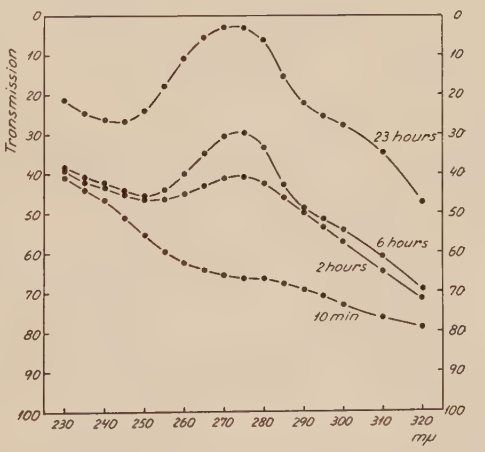
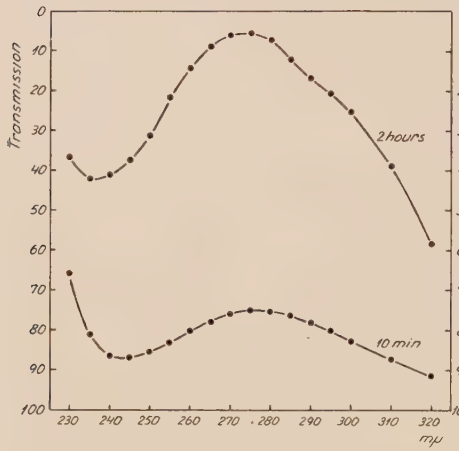


Fig. 6 (left). Ultraviolet absorption spectra of D-glucosamine-hydrochloride solutions at pH 7.0 and 37°C measured at different time intervals.

Fig. 7 (right). Ultraviolet absorption spectra of D-glucosamine-hydrochloride solutions at pH 7.0 and 37°C measured at different time intervals.

As demonstrated above, solutions of D-glucosamine hydrochloride in Gey's balanced salt solution possessed a tumour growth inhibiting and cell damaging effect. It was therefore of interest to determine whether ultraviolet absorption spectra similar to that above in aqueous solutions was also formed in Gey's solution.



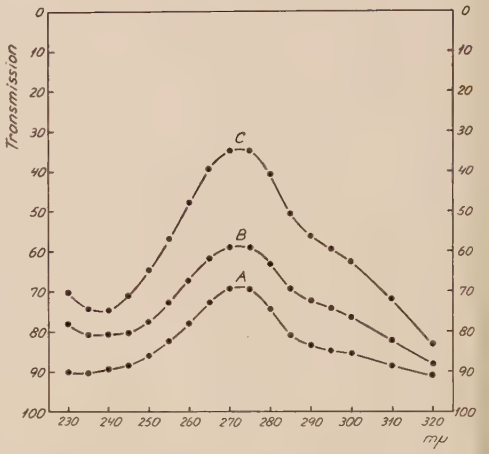
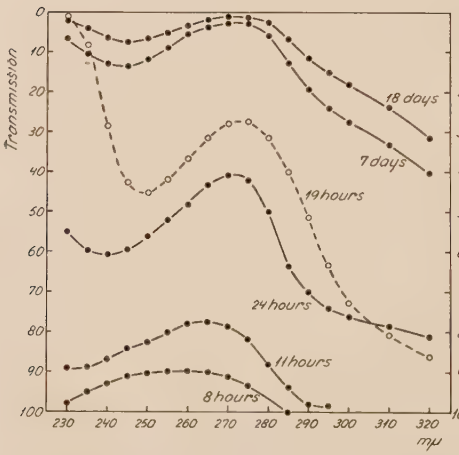


Fig. 8 (left). Ultraviolet absorption spectra of D-glucosamine-hydrochloride solutions at pH 13 and 37°C measured at different time intervals.
Fig. 9 (right). Ultraviolet absorption spectra of D-glucosamine-hydrochloride solutions in isotonic solutions of (A) NaCl, (B) KCl, (C) Locke-Ringer at pH 6.6 measured after 19 hours incubation at 37°C.



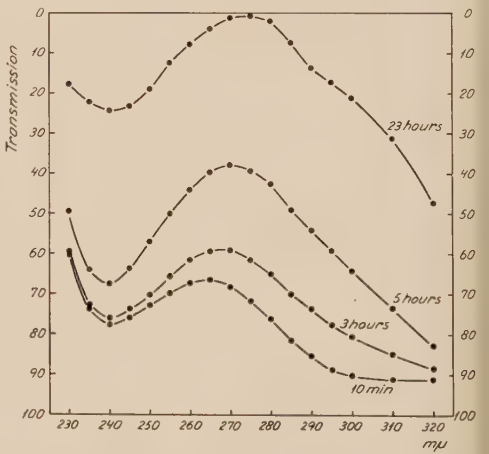


Fig. 10 (left). Ultraviolet absorption spectra of D-glucosamine-hydrochloride solutions in Gey's balanced salt solution at pH 6.5, measured after different time intervals. The curve in a broken line – ○ – ○ – ○ – ○ – represents a solution withdrawn from a tissue culture experiment.
Fig. 11 (right). Ultraviolet absorption spectra of D-glucosamine-hydrochloride solutions in medium 199 at pH 6.4 measured at different time intervals.

Fig. 10 indicates the same absorption maximum at 272 mμ. The glucosamine solution with a pH of 6.5 was chosen, since the most acid pH of the highest dose of glucosamine in tissue culture was pH 6.5. The – ○ – ○ – ○ – ○ – curve in Fig. 10 shows the curve for the medium at the end of a 19-hour tissue culture experiment. The curves demonstrate that a similar concentration of the absorbing compound is also formed in Gey's balanced salt solution without the presence of cells. It further seems evident that the absorbing compound possesses a relatively high stability, since a similar absorption is found after 18 days at 37°C. After standing for 6 months in the refrigerator, at pH 5.0, 6.5, and 8.0 higher concentrations of the same component are present.

The toxic effect of D-glucosamine on the tumour cells in medium 199 has been noted to be stronger and to occur more rapidly than in Gey's medium. The spectrophotometric study of the behaviour of glucosamine in this medium was therefore undertaken. Medium 199 was used in dilutions of 1–10, since it was impossible to adjust the original solution to the Beckman apparatus due to the strong absorption present.

The curves in Fig. 11 demonstrate at pH 6.4 an absorption maximum at 270 mμ after 3–5 hours, and at 275 mμ after 23 hours. It is significant that the reaction underlying this change occurs more rapidly in medium 199 than in Gey's or other solutions employed. Whether this is due to the catalytic activity of some of the components of medium 199 is yet unknown, though likely.

DISCUSSION

A study of the comparative effect of glucose and D-glucosamine on human carcinoma cells in tissue culture is presented, using an improved technique for estimating inhibition of tissue culture growth.

The results indicate that the tumour cells could tolerate large amounts of glucose, but were killed by relatively small amounts of glucosamine. Experiment 1 showed that amounts of glucosamine ranging from 1–50 mg/ml produced cell death and growth inhibition. The same amounts of glucose produced growth within the normal range, excepting a 40 per cent reduced growth rate at 50 mg/ml. The 2nd and 3rd experiments showed death of cells for both glucose and glucosamine at 500 mg/ml, and a normal growth rate in glucose solutions at 50 mg/ml and 5 mg/ml. In Gey's medium and medium 199 a normal growth rate with glucosamine was attained between .05 micrograms and 50 micrograms. Cell damage from glucosamine could be seen after 7–8 hours, and the cells at the actively growing edge were more sensitive than cells in a resting condition. Dormant cultures [7] were not sensitive.

Additional results, not mentioned in this paper, indicate that the inhibition and cell death could not be attributed to the hydrogen ion concentration,

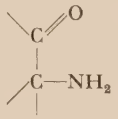
since no significant differences could be found at pH ranges from 6.5–8.5. Competition between glucose and glucosamine for an enzyme could not be demonstrated by adding increasing amounts of glucose to cultures treated with glucosamine in Gey's balanced salt solution without glucose. The cell damage seemed to depend on the absolute amount of glucosamine rather than on the proportionate amounts of glucose and glucosamine, as stated by Woodward and Hudson [14]. However, further work is in progress for studying these relationships.

The system introduced here seems useful in studying biological relationships between cells and damaging agents. A further advantage of the system is the possibility of prolonging the study indefinitely, since the measurements do not necessitate sacrificing the cultures; and from time to time, tissue can be removed for cytological study and record keeping.

Expressing tissue culture growth ratios has inherent difficulties, which can be solved to some extent by using the cells and technique most suitable to the investigation. Stable, uniform strains of cells circumvent the irregularities of adaptation encountered in using fresh explants or exudates. An unstable cell population introduces difficulties in evaluating the results in terms other than morphological. An all or none effect is not difficult to demonstrate, but relatively few tissue culture problems can be so easily dispensed with.

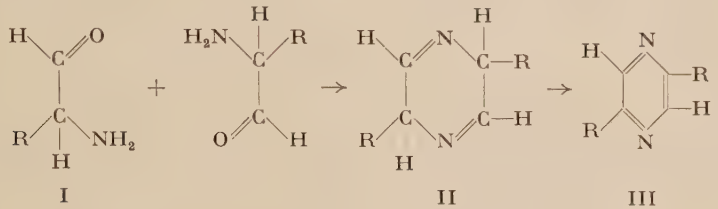
It is interesting to note that Ely, Tull, and Schanen [5], using 1 mg/ml glucosamine, report a 1.61 mitotic index for chick heart fibroblasts, or approximately 50 per cent of the normal control. The same amount of glucosamine in the present study produced a growth index of .44 in Gey's medium and .19 in medium 199, or 44 and 19 per cent of normal growth values. It is also interesting to note that investigations [2] concerning the effects of bacterial polysaccharide indicate that the *Seratia marcescens* polysaccharide produced maximum damage to cancer cells after about 6–7 hour in animal experiments, and that resting cells were less sensitive than dividing cells.

D-glucosamine belongs to the class of α-amino carbonyl compounds.



Such compounds have been described earlier by Erlenmeyer, Lehmann *et al.* [6, 2] as inhibiting the regeneration of the tails of *Xenopus* larvae. One of the characteristics for this type of chemical structure is the ease with which

dimerization occurs. When present as a free base, or in the presence of high hydroxyl ion concentrations, cyclic compounds of the pyrazine type are formed. D-glucosamine thus acts in the following manner:—



I II III

It is likely that the intermediate dihydropyrazine derivative II is formed first, which is then further oxidized to the aromatic pyrazine derivative. Under strongly alkaline conditions, this reaction occurs very quickly (Fig. 8). With the exception of a few highly aromatic di-hydropyrazine derivatives, it has not been possible, however, to isolate the intermediate products.

Whether these types of reactions, or only the steps I–II, are actually involved in producing the biological effects described above has yet to be demonstrated. There is some evidence, such as the appearance of the absorption maximum at 272 mμ even at a slightly acid pH, the stronger and more rapid action in medium 199, parallel to the more rapid appearance of this maximum at 272 mμ, which supports this hypothesis. It must be pointed out, however, that only the isolation of the hypothetical intermediate product free from glucosamine can clarify this problem.

There is furthermore the possibility that D-glucosamine is the reactive component. It could evoke its toxic effects by interfering with metabolic processes for example, as an antimetabolite for glucose (this is not supported by preliminary inhibition studies; however, the inhibition is about twice as strong in medium 199, which contains one-half the amount of glucose present in Gey's medium). Whether this is due to a relatively larger amount of glucosamine in relation to glucose, or to a higher amount of the substance with the absorption maximum at 272 mμ, is not evident.

SUMMARY

The toxic and growth inhibiting effects of D-glucosamine on human tumour cells in tissue culture has been studied, and a method for the evaluation of growth inhibition is described. The tissue culture technique introduced seems useful in demonstrating growth relationships.

Chemical changes including formation of a substance with an absorption

maximum at 272 mμ occurring in the glucosamine molecule under these experimental conditions were analyzed by spectrophotometric studies in the ultraviolet, stressing the lability of the glucosamine molecule under various conditions.

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EFFETS DU RÉACTIF DE SCHIFF SUR LES ŒUFS EN SEGMENTATION DU RAT ET DE LA SOURIS

ÉTUDE QUALITATIVE

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Reçu le 7 mai, 1955

Le réactif de Schiff a un effet positif sur le cytoplasme si celui-ci a été fixé dans un mélange à base de sublimé. Le complexe plasmalogène ainsi décelé présente une localisation et une évolution intéressante. Sa synthèse cyclique est, comme celle des mucopolysaccharides, en relation étroite avec les divisions cellulaires. L'hydrolyse préalable à l'action du Schiff ne révèle de DNA que dans les noyaux. Une étude comparative des divers fixateurs a été faite, pour aboutir à une méthode convenant pour la photométrie du DNA. Chemin faisant, diverses observations cytologiques ont été rencontrées.

Au cours d'essais cytochimiques antérieurs, il a été remarqué [6] que si des œufs de Rat divisés en VIII blastomères étaient traités comme pour le Feulgen, mais en omettant l'hydrolyse, il se produisait une coloration violacée dans les blastomères du groupe enveloppant, encore appelé ventral [7]. Il a été également observé alors que la réaction de Feulgen, appliquée après fixation au formol calcique de Baker ou au S.P.W.M. d'Izquierdo [12] montrait une appréciable différence d'intensité entre les pronucléi d'une part, les noyaux des stades IV et VIII d'autre part; occasionnellement, une coloration violacée du cytoplasme suggérait l'existence, dans celui-ci, d'une réserve éventuelle de DNA. Il y avait là plusieurs raisons incitant à examiner méthodiquement les effets du réactif de Schiff dans les diverses conditions susceptibles d'affecter cette réaction. Il s'agit donc d'une étude où la mise au point d'une technique a une part prépondérante, mais qui a cependant conduit à quelques constatations d'un réel intérêt théorique.

MATÉRIEL ET TECHNIQUE

Les objets examinés ont été les oocytes obtenus par dilacération des follicules et élimination mécanique des cellules coronales, les œufs tubaires tant vierges que fécondés (indivis ou segmentés en II, IV, VIII blastomères), les morulas ou les très jeunes blastocystes déjà parvenus dans les cornes utérines (5^{e} jour chez le Rat).

40 ♀ de Rat et 4 ♀ de Souris ont été utilisées, fréquemment avec examen successif des deux complexes génitaux à quelques heures, ou à 1 jour, d'intervalle, soit, en tout, 62 groupes de 2 à 5 œufs.

Absence de faux plasmalogène

Après immersion directe des œufs vivants dans le réactif de Schiff, il ne se développe aucune coloration perceptible après report dans le bisulfite de Na acidifié. Il en est de même lorsque les œufs sont d'abord traités par un fixateur dépourvu de sublimé, tel le formol calcique. *Aucune indication en faveur d'un faux plasmalogène* n'a été relevée.

Recherche du plasmalogène vrai

L'attention s'est ainsi tournée vers la recherche du *plasmalogène* vrai, se manifestant après fixation « courte » dans un fixateur contenant du $HgCl_2$. Des raisons de commodité[1] ont fait choisir, à cause de sa stabilité, et de son excellent pouvoir fixateur, le mélange S.P.W.M. [12], mélange à base de sublimé, d'acide phosphotungstique et de molybdate d'ammonium. On pourrait cependant lui reprocher de provoquer une rétraction appréciable des œufs, mais cette particularité n'affecte en rien les faits qui nous intéressent ici.

Un séjour de 5 à 10 minutes dans ce fixateur suffit amplement à susciter les conditions d'où dépend la réaction du plasmalogène. Elles ne semblent pas modifiées par une durée de quelques heures.

La coloration caractéristique, d'un rouge vineux, se développe dès que les œufs ainsi fixés sont immergés dans le réactif de Schiff à la température ordinaire. Le délai observé parfois pour des cellules profondes de la morula ou du blastocyste paraît simplement correspondre aux quelques secondes nécessaires à la pénétration du complexe fuchsinique. La prolongation du séjour dans le Schiff reste sans conséquence, de même que l'élévation de la température à 37°.

Une période de 20 à 30 minutes a été adoptée pour l'action du Schiff. Lors du report dans le métabisulfite acidifié (63 cc $Na_2S_2O_5$ à 5 % + 3 cc HCl · N), la coloration s'intensifie légèrement et une sorte de différenciation se produit. Ce changement est simplement dû à l'élimination de la coloration jaunâtre propre au réactif de Schiff.

Il a été spécialement examiné si la réaction observée à cette phase des opérations, tant sous la loupe binoculaire, qu'au microscope, entre lame et lamelle, était affectée par d'autres conditions que la présence du Sublimé dans le fixateur. En particulier, il a été recherché si l'hydrolyse à diverses températures et à diverses concentrations de la solution de HCl entraînerait une modification. Rien de décisif n'a été constaté. Parfois, au moment où les œufs étaient plongés dans la solution sulfureuse, les œufs précédemment hydrolysés ont paru plus colorés que les témoins, mais il s'agit d'un effet fugace. A part cette nuance, on n'obtient pas d'effet différent sur l'aspect du cytoplasme, même après l'hydrolyse révélatrice du DNA (5, 8, 12 min. dans HCl · N à 60°C), mais on aperçoit naturellement, dès le traitement par le Schiff, la « réaction nucléale ». Dans la technique usuelle qui aboutit au montage dans le baume du Canada (naturel ou synthétique), c'est au cours du passage dans les alcools et le

[1] Il serait indiqué de reprendre cette étude après fixation au formol calcique et légère hydrolyse, conformément aux vues de J. F. Danielli ([10], p. 80).

toluol que la coloration cytoplasmique s'évanouit entièrement. Cette constatation est conforme à l'interprétation classique de la réaction de Feulgen. L'observation *princeps* rappelée plus haut, et qui concernait des œufs fixés au formol calcique, n'est pas entièrement élucidée par l'enquête actuelle (voir note p. 100).

La solubilité du complexe plasmalogène dans les solvants des graisses est effectivement très marquée. Si, au sortir du SO^2 — nous allons revenir sur la durée à adopter pour ces lavages — on monte les œufs sur lame par le procédé à l'albumine fraîche (voir ci-dessous), le simple passage dans les vapeurs d'alcool pendant les 60 à 100 secondes nécessaires pour obtenir la coagulation suffit déjà à atténuer la réaction. Si l'on procède par les bains usuels d'alcool et de toluol, elle s'évanouit entièrement bien avant l'addition du baume. Au cours de ces étapes, s'il n'y a pas eu d'hydrolyse, ou seulement une hydrolyse trop faible pour affecter le DNA, aucune coloration ne s'observe dans les noyaux.

Vu cette solubilité, il a été tenté d'améliorer les conditions d'observation grâce à un montage direct dans la gomme-sirop ou dans la gomme arabique. Il est immédiatement apparu que même après trois bains d'une demi-heure dans la solution saturée de SO^2, le passage dans le milieu de montage entraînait une coloration rouge des objets. Celle-ci apparaissait d'abord dans la membrane, puis s'étendait aux corps cellulaires. De toute évidence, des traces du mélange de Schiff s'étaient maintenues dans les objets et elles étaient suffisantes pour réagir avec les groupes aldéhydiques présents dans le milieu de montage. Toutefois, si l'on prolonge les lavages en utilisant 5 bains répartis sur 15 à 20 h (dont un pendant la nuit), la surcoloration cesse de se produire. Par la même occasion, il a été constaté que si l'on prolonge davantage ces bains, la réaction du plasmalogène s'atténue assez rapidement (au delà de 24 h, elle devient remarquablement faible) comme si le complexe coloré se redissolvait dans la solution saturée de SO^2.

Même en prenant ces précautions, la stabilité des préparations ainsi montées, notamment à la gomme arabique, reste précaire. Elles permettent néanmoins des observations aux plus forts grossissements, avec l'avantage que sous l'objectif à immersion les mouvements de la vis micrométrique font tourner les œufs, de sorte qu'on en saisit tous les détails.

La *localisation* du plasmalogène a également été précisée à l'aide des centrifugations. Celles-ci ont été effectuées sur le complexe génital intact selon la technique utilisée en 1953 ([8], p. 319 : centrifugeuse Sörvall, vitesse max. 12.000 tours minute, accél. 19.500 *g*) avec des temps de 7 à 15 min. pour la période de mise en marche progressive, 30′ pour la période de régime, 7′ pour la période d'arrêt progressif. La recherche des œufs et leur traitement ont été faits comme de coutume.

Recherche du DNA

Deux préoccupations ont guidé ces essais : déceler, si possible, un éventuel constituant cytoplasmique réagissant avec le Schiff; obtenir une réaction nucléale permettant les mesures photométriques.

Absence de DNA cytoplasmique décelable par ces méthodes.—Sans doute, dans notre expérience antérieure, la majorité des Feulgen a-t-elle montré, tant dans les oocytes que dans les œufs en segmentation, un cytoplasme parfaitement incolore. Les quelques exceptions pouvaient *a priori* s'interpréter par un phénomène de

recoloration du complexe sulfo-fuchsinique insuffisamment lavé. Il convenait cependant de considérer s'il n'existerait pas une réserve cytoplasmique diffuse, notamment sous la forme d'un corps du type "acide thymique" qui serait habituellement éliminé par l'hydrolyse, ou masqué par le plasmalogène. Il convient pour cela d'éviter l'emploi des fixateurs contenant du sublimé, puisque la coloration du plasmalogène masquerait la réaction éventuelle.

Des œufs (R. 636, stades I et II) ont été fixés 15 sec. aux vapeurs de OsO^4 à 2 %, traités par le Schiff (30 min.) puis lavés aux bains sulfureux et montés sur lame dans l'albumine; un passage prolongé dans les alcools et le toluol a précédé le montage dans le baume. Les objets ainsi traités étaient totalement incolores, tant leur cytoplasme que leur noyau.

On s'est alors demandé si la présence du plasmalogène n'empêchait pas l'accès du réactif à des matériaux préexistants, qu'il s'agisse des mêmes hypothétiques complexes du type acide thymique, révélables sans hydrolyse, ou de DNA préformé, qui exigerait l'hydrolyse. C'est pourquoi, dans un nouveau test, des œufs (R. 626, st. IV) ont été fixés au S.P.W.M., délipidés par un séjour d'1 h dans l'alcool absolu, soumis au Schiff, lavés aux bains sulfureux, et montés dans la gomme-sirop: ils se sont montrés entièrement négatifs. D'autre part, un œuf du stade II (R. 629) a été également fixé par le S.P.W.M., monté sur lame à l'albumine, transporté dans les alcools et le toluol où il est resté 3 h, redescendu, ainsi délipidé, jusqu'à l'eau, soumis à 7 min. d'hydrolyse à 64°C, puis au Schiff, lavé dans les bains sulfureux, finalement inclus dans la gomme arabique en l'extrayant du gâteau d'albumine, pour améliorer la visibilité : seuls les noyaux étaient, comme il se devait, positifs. Un essai analogue a été répété avec 5 œufs du stade IV (R. 632), après fixation parallèle au S.P.W.M. et aux vapeurs de OsO^4 à 2 % (15″), délipidation de 40 min. dans le toluol à 50°C, hydrolyse de 5 à 10 min. à 59°5, 30 min. de Schiff à 24°C; le résultat a été examiné dans les bains sulfureux, dans la gomme arabique, dans le baume Harleco, toujours sans aucun indice de réaction positive dans le cytoplasme.

Il est donc permis de conclure que dans la limite des moyens d'observation utilisés *il n'existe pas, dans le cytoplasme des œufs tubaires du Rat, de constituant du type acide thymique ou DNA*. Le seul matériel que réagisse avec le Schiff est le plasmalogène vrai, dont la révélation suppose une fixation adéquate[1] et la non-utilisation de solvants des graisses. Quant à la coloration violacée parfois observée dans le cytoplasme ([6], p. 1350) elle doit probablement être mise au compte d'une recoloration de la fuchsine, à cause d'un lavage alors insuffisant dans les bains sulfureux.

Mise en évidence du DNA nucléaire.—Sous ce rapport, les informations se résument en ceci :

1°) *Montage sur lame à l'aide de l'albumine fraîche.* La recette donnée précédemment ne suffit pas toujours à éviter les décollements après l'hydrolyse. Il y a avantage à tenir compte de l'amélioration rencontrée par J. Mulnard au cours de ses recherches

[1] Ou d'après Danielli [9], en l'absence de sublimé dans le fixateur, une très légère hydrolyse de démasquage.

sur les phosphatases alcalines. Epurer le blanc d'œuf frais de toute condensation filamenteuse; diluer la partie fluide en ajoutant $\frac{1}{4}$ d'H_2O seulement; filtrer sur papier. Pour monter les œufs, placer sur une lame une goutte de 3 à 4 mm de diamètre, y injecter les objets, réduire l'épaisseur maximum de la goutte à $\pm \frac{1}{2}$ mm. Placer ce porte-objet, la goutte *au-dessus*, dans une boîte de Pétri contenant un fond d'alcool à 90°, laisser exposé 60 à 80 sec. aux vapeurs d'alcool, de manière que la périphérie de la goutte commence à se coaguler. Résorber alors l'excès d'albumine en groupant les objets bien au centre, renverser le porte-objet dans la boîte de Pétri, la goutte *en dessous*, de façon que la coagulation finale se fasse en 5 à 10 sec. Reporter sur le champ, en évitant la dessication, dans l'alcool à 70°.

Lorsque, la réaction de Feulgen étant effectuée, on arrive dans le baume, on peut, si on le désire, extraire les œufs du gel d'albumine, afin d'obtenir la meilleure visibilité possible. Cette manœuvre, répétée fréquemment dans la période de mise au point, notamment pour s'assurer de l'absence de toute réaction dans le cytoplasme, a été finalement abandonnée lorsque la technique définitive a été atteinte; la trame hyaline d'albumine ne nuit en rien ni à la lecture des préparations ni aux mesures photométriques.

2°) *Recherche du fixateur préférable pour la photométrie. Les vapeurs* de OsO^4 conservent, du point de vue qualitatif, les qualités précédemment signalées [2, 3, 14]. Dans une gouttelette pendante de Tyrode, le temps optimum d'exposition aux vapeurs de la solution à 2 % est de 15″. Avec un temps d'hydrolyse de 8 min., la finesse des détails nucléaires est vraiment idéale; les filaments chromosomiaux, leurs chromioles, le sertissage des nucléoles sont d'une netteté parfaite à toutes les phases des noyaux quiescents. L'inconvénient est la teinte ocre du cytoplasme, qui oblige à se référer, pour la comparaison photométrique, à un « blanc » déjà sérieusement coloré. Il a donc fallu, pour notre propos actuel, écarter cet excellent fixateur.

La fixation au S.P.W.M. a également de sérieuses qualités. Les aspects nucléaires (voir [6], fig. 14), dont l'optimum répond à 10 min. d'hydrolyse à 60°C, sont toutefois un peu moins précis qu'avec l'Osmium. Ils sont aussi affectés par la rétraction des corps cellulaires. Il s'y ajoute que le cytoplasme conserve une teinte de fond bleutée qui n'est pas favorable à des mesures photométriques délicates. Ce mélange n'a donc pu être retenu.

On s'est alors tourné vers le *formol calcique*, avec lequel des images remarquables avaient déjà été signalées (ibid., fig. 10, 11, 15, 16). Ces qualités ne se sont pas démenties, et quelques-uns des résultats signalés ci-dessous ont été obtenus grâce à ce fixateur (fig. 7, 8, 9). Le temps optimum d'hydrolyse est de 12 min. à 60°C. Il n'y a aucune coloration de fond. Cependant, il se produit dès l'immersion dans le fixateur, un gonflement de l'œuf, surtout important aux premiers stades, précisément ceux pour lesquels le volume considérable des noyaux abaisse déjà la concentration du DNA au point de compromettre la précision des mesures. De plus, l'imprégnation par de l'aldéhyde formique oblige à un lavage « prolongé », qui laisse quand même subsister une arrière-pensée.

Aussi faut-il se louer de la coincidence qui a fait remarquer par J. Mulnard l'excellente qualité de la fixation obtenue par le simple *alcool à 85 % refroidi à 2°C.* Cette propriété tient vraisemblablement au fait que la goutte de Tyrode contenant les œufs s'étale au fond du récipient et persiste quelques instants avant d'être graduellement pénétrée par le flux d'alcool. Les conditions ainsi réalisées sont vraiment opti-

males. Une seule substance fixatrice intervient, qui est précipitante des acides nucléiques; une prolongation modérée de son action est sans inconvénient; grâce à la pénétration graduelle, il n'y a ni rétraction sensible, ni fuite perceptible dans les noyaux[1]; aucune manœuvre complémentaire n'est nécessaire; le plus souvent, la membrane s'élimine facilement sans que les blastomères se détachent les uns des autres; la conservation des structures nucléaires le cède à peine en finesse à celle qu'assurent les vapeurs osmiées. Enfin, pour les mesures photométriques, le même fixateur convient parfaitement pour les empreintes de foie qui fourniront les valeurs de référence pour les œufs du même animal [9]. La durée optimale d'hydrolyse a été recherchée par des essais menés parallèlement d'une part sur des montages contenant des œufs, des oocytes revêtus de cellules folliculeuses, des fragments de muqueuse tubaire, et d'autre part sur des empreintes de tissu hépatique du même animal. Pour le 1er groupe de matériaux le résultat d'hydrolyses de 4, 6, 8, 10, 12 min. a été apprécié au jugé; pour les empreintes de foie, la comparaison a été basée sur des mesures photométriques faites par J. Pasteels. La conclusion concordante a été que le temps optimum était de 8 min. à 60°C. Cette recette a permis d'obtenir des résultats d'une constance très satisfaisante, à condition d'utiliser un Schiff ne datant pas de plus d'une semaine.

Par cet acheminement, la *technique actuellement optimale* du Feulgen sur l'œuf de Mammifère s'est établie ainsi:

Fixation à l'alcool à 85 % refroidi à 2°C, de 30 min. à 12 h (ou plus). Transfert facultatif dans l'alcool absolu. Report dans H_2O, sur laquelle les œufs flottent; à l'aide du fil de platine, on les ramène au fond, où leur membrane se détache aisément (sauf parfois pour les œufs indivis). Montage sur lame selon le procédé indiqué plus haut. Hydrolyse de 8 min. à 60°C — Schiff — bains sulfureux prolongés (5 bains en 10 à 15 h). Montage au baume Harleco.

La centrifugation a parfois été utilisée, dans la recherche du DNA, avec la technique signalée plus haut.

Les illustrations ont été établies d'après des croquis de protocole et d'après des photos prises au Leica au cours des observations.

OBSERVATIONS CYTOLOGIQUES

La recherche dont il vient d'être rendu compte a successivement permis de saisir la localisation et l'évolution du plasmalogène vrai, de préciser, chemin faisant, l'organisation générale du cytoplasme dans l'œuf indivis et dans les blastomères, de mettre en évidence une modification d'ordre physique dans les noyaux. A ces constatations essentiellement qualitatives se relient

[1] Dans le cytoplasme, J. Mulnard n'a constaté qu'un déplacement insignifiant du glycogène [16].

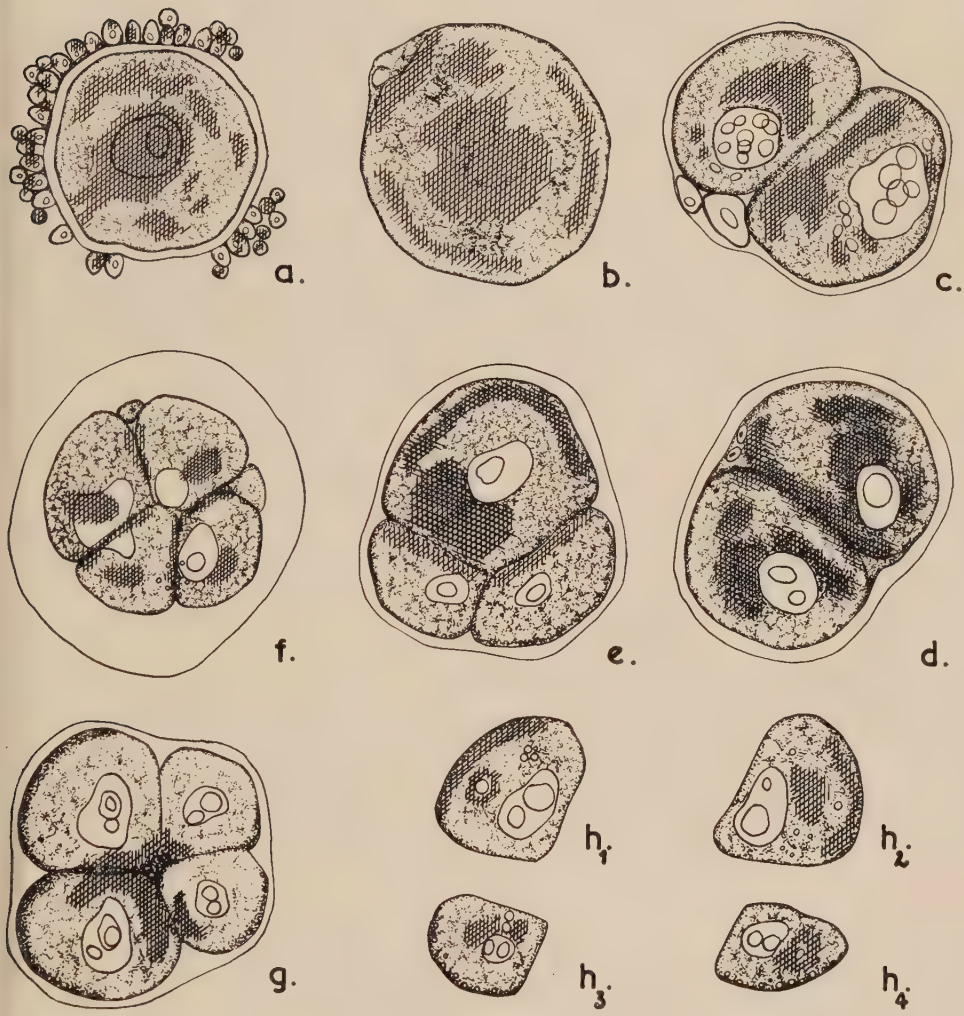

Fig. 1. Distribution des grains mitochondriaux (pointillés) et du plasmalogène (traits entrecroisés) dans l'œuf normal du Rat jusqu'au stade IV. *a*) Oocyte de 1er ordre extrait d'un follicule, avec quelques cellules folliculeuses résiduelles également positives; *b*) œuf fécondé indivis, âgé d'environ 10 h; *c*) œuf divisé en 2 blastomères, au début du 2^{d} jour (âge 30 à 35 h); *d*) œuf divisé en 2 blastomères, au début du 3^{e} jour (âge 50 à 55 h); *e*) stade intermédiaire à 3 blastomères, avec la surcharge du blastomère encore indivis; âge env. 58 h; *f*) stade à 4 blastomères, au 3^{e} jour après-midi, âge environ 58 à 60 h; *g*) stade à 4 blastomères, même âge, avec disposition symétrisée (taille des blastomères, granulations, plasmalogène, nucléoles); h_1 à h_4) détail des 4 blastomères d'un œuf frère du spécimen *f*, et dont les blastomères ont été séparés par dissection après la réaction. Etude à l'immersion. *Détection de sphérules présumées d'origine nucléolaire*, dont certaines enclavées dans l'amas de plasmalogène.

les mesures de la richesse relative en DNA et l'interprétation de certaines anomalies du clivage, aspects quantitatifs exposés avec J. Pasteels dans un mémoire distinct [9].

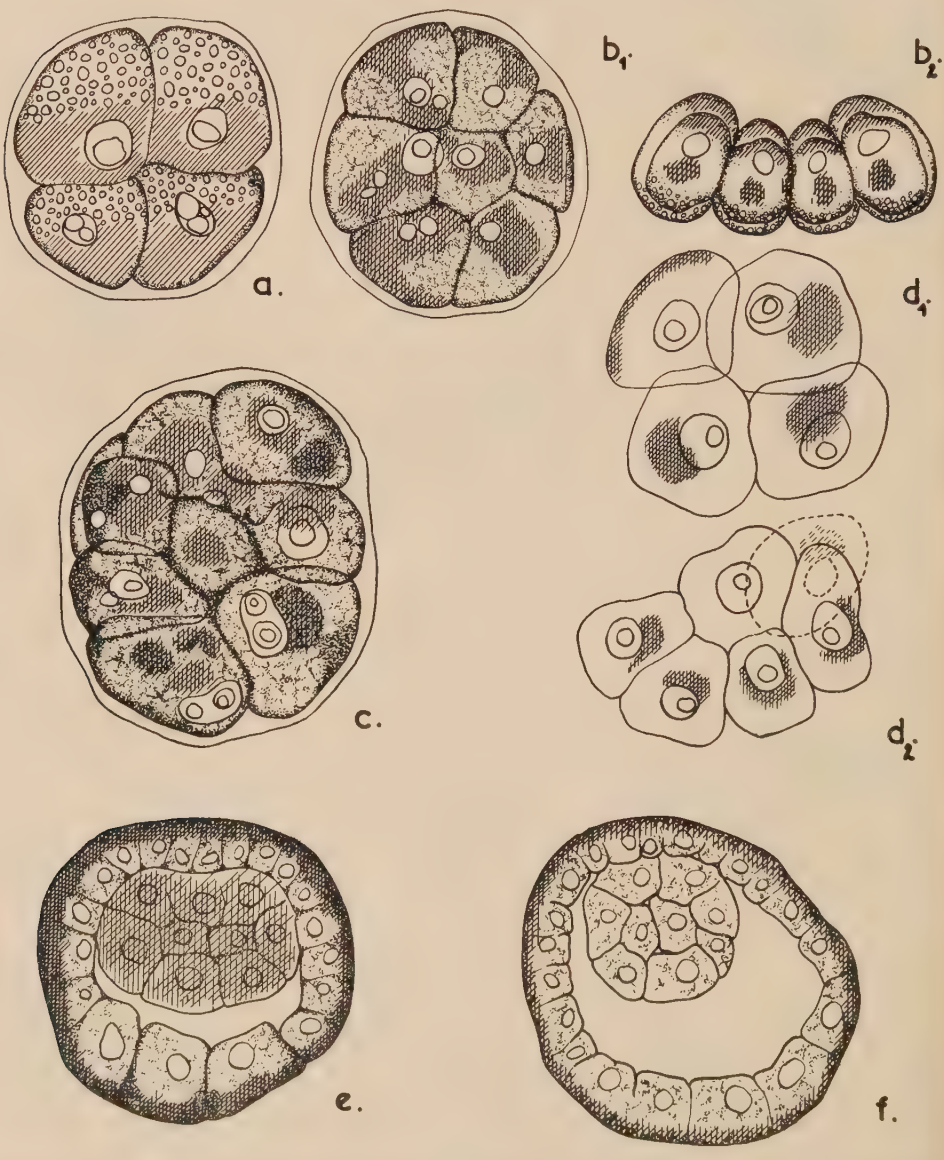

Fig. 2. Distribution des grains mitochondriaux (pointillés) et du plasmalogène (traits entrecroisés) dans l'œuf normal du Rat, du stade IV au jeune blastocyste.

Le plasmalogène vrai dans les œufs de Rat et de Souris

On sait que, chez ces deux espèces, la constitution de l'œuf présente des différences déjà perceptibles *in vivo*, et plus encore après les diverses réactions cytochimiques (cf. [5]). On sait aussi que chez le Rat la disposition des blastomères en une *placula*, du moins dans le type dit régulier, facilite grandement l'examen. Ces différences ont leur incidence sur l'observation du plasmalogène. Qu'il s'agisse de l'une ou l'autre espèce, une description détaillée peut être évitée, pour qui a présentes à l'esprit la distribution et l'évolution de la métachromasie au bleu de toluidine, telle que Izquierdo vient de la préciser pour le Rat [12, 13], telle aussi que je l'ai décrite (obs. de E. Boulanger) dans l'œuf de Souris ([5]). La topographie des zones positives au Schiff (coloration d'un rouge vineux) se retrouve exactement la même, sous réserve que *la réaction n'intéresse pas les granulations métachromatiques mais bien l'hyaloplasme* dans lequel celles-ci sont enrobées. Cette localisation particulière a été établie en examinant des blastomères que l'on avait réussi à séparer les uns des autres, et que l'on comprimait sous l'objectif à immersion, de façon à les faire pivoter d'abord, à les écraser ensuite.

Chez le Rat, la réaction se présente comme le résume la fig. 1. Dans l'oocyte extrait du follicule (*a*), c'est un nuage rose entourant la vésicule germinative, et parfois aussi une couche subcorticale enrobant les mitochondries présentes à ce niveau. Dans l'œuf fécondé depuis une dizaine d'heures (*b*), on voit un large nuage entourant les pronucléi, autour de celui-ci une zone aréolaire plus claire et enfin une garniture corticale non uniforme et souvent discontinue. Au stade II, l'aspect varie quelque peu selon qu'il s'agit d'œufs assez récemment divisés (*c*), âgés d'une trentaine d'heures, ou (*d*) de ceux qui sont à la fin de cette longue intercinèse (50 h et plus). Dans les deux cas, il se forme des nuages vineux dans l'aire périnucléaire, ainsi que des traînées corticales et une forte bande répondant à la cloison interblastomérique. La différence causée par l'âge est que, *dans les blastomères se préparant à la prophase, la réaction devient plus intense, surtout dans la zone périnucléaire.* Cette surcharge est entièrement

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a) Exemple de stade IV à symétrisation perceptible; les deux blastomères dorsaux contiennent également, vers leur pôle végétatif une portion lacunaire (cercles), mais elle est plus réduite que dans les blastomères ventraux; la partie dense (hâchures simples) a la disposition inverse. Les noyaux de ceux-ci (nucléoles unifiés) révèlent qu'ils se sont constitués les premiers, aux dépens du plus gros blastomère d'un stade II inégal. Ni le plasmalogène ni les mitochondries ne sont représentés.

b) Stade à VIII blastomères, œuf du 4^{e} jour au matin (environ 78 h); b_1 vue du pôle animal; b_2 vue latérale, pôle végétatif en bas, avec les parties denses (hâchures simples) et lacunaires.

c) Stade à X blastomères, œuf du 4^{e} jour après-midi (env. 82 h). Prépondérance du plasmalogène dans les gros blastomères ventraux. La localisation intergranulaire a pu être constatée. Léger enrichissement par rapport à *b*.

d) Œuf frère du précédent, également divisé en X blastomères grâce aux mitoses plus précoces des blastomères dorsaux. En d_1, les 4 blastomères ventraux, petits, très chargés de plasmalogène, en d_2 les 6 dorsaux, moins riches, surtout 3 d'entre eux (effet des divisions toutes récentes). Les mitochondries n'ont pas été représentées.

e) Blastocyste du 5^{e} jour ($\pm$ 100 h). Plasmalogène mêlé aux grosses granulations corticales de la couche enveloppante, légère manifestation dans le groupe enveloppé.

f) Blastocyste frère du précédent, à cavité plus dilatée. Plasmalogène uniquement dans la couche périphérique.

parallèle à ce que montre la coloration vitale au bleu de toluidine (fig. 6, avec légende explicite). La même différence est bien mise en évidence par l'observation des stades III, où l'on a, côte à côte (*e*), deux blastomères « IV » jeunes et un blastomère « II » âgé. De même que celui-ci sera plus chargé de plages métachromatiques, il montrera

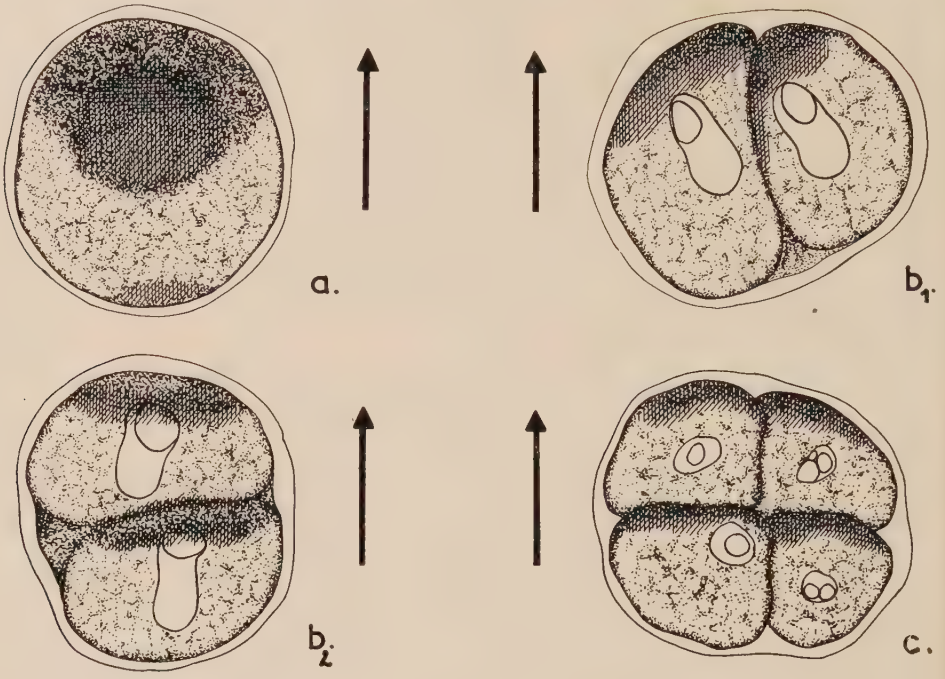


Fig. 3. Effets de la centrifugation (15 + 30 + 7 min.) sur la distribution des mitochondries (pointillés) et du plasmalogène (traits entrecroisés) dans l'œuf du Rat. La flèche indique la direction centrifuge.

a) Stade indivis avancé. Remarquer une petite calotte centripète.
b) Stade à 2 blastomères, âge environ 35 h. Dans un cas, b_1, la centrifugation s'est faite parallèlement au plan de clivage, dans l'autre, b_2, perpendiculairement.
c) Stade à IV blastomères. La sédimentation se fait plus difficilement et ne dépasse pas ce degré.

au Schiff une réaction nettement plus forte, surtout près du noyau et le long de la membrane profonde. Nous reviendrons sur la signification de ce fait.

Au stade à 4 blastomères, on trouve, tout au moins au début de l'intercinèse, une réaction périnucléaire ou juxtanucléaire de forme et d'étendue un peu variable, un semis de points dispersés dans le cytoplasme, des traînées corticales et des condensations le long des interstices profonds (fig. 5, fig. 1, *f*). Parfois, on a l'occasion d'observer une organisation symétrisée, allant jusqu'à correspondance des nucléoles dans les deux petits blastomères ventraux et dans les deux gros dorsaux (*g*, h_1 à h_4).

Après la division (progressive!) en 8 blastomères (fig. 2, b_1), la réaction présente la même intensification brusque que pour la métachromasie au bleu de toluidine.

Une sorte de sphère rouge apparaît dans chaque blastomère, et du côté ventral, elle va rejoindre une lame corticale souvent discontinue. Ces aspects se retrouvent, au moins aussi intenses, pendant la progression de VIII à XVI blastomères (*c*).

Enfin, dans les blastocystes recueillis au 5^{e} jour, la positivité de l'ensemble s'at-

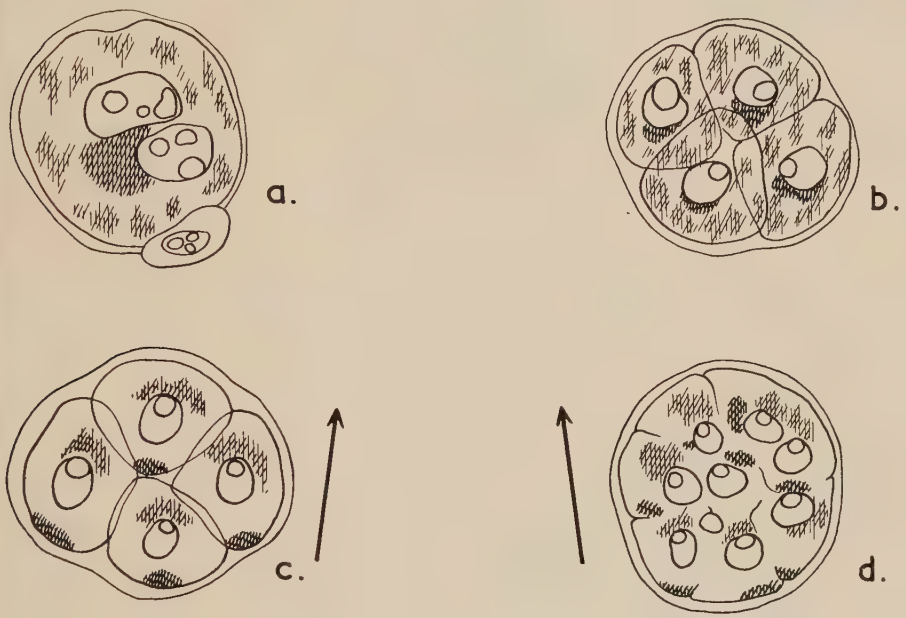


Fig. 4. Réaction du plasmalogène (traits entrecroisés) dans les œufs de Souris normaux et centrifugés.

a) Œuf normal indivis (1er jour, après-midi). Pronucléi alignés selon l'axe de polarité. Maximum de réaction dans la zone internucléaire, d'un côté de l'axe An–Vg.
b) Œuf normal à 4 blastomères (3^{e} jour). Réaction plus diffuse que chez le Rat.
c) Effet de la centrifugation (15 + 30 + 7 min.) sur les œufs frères de *b*. La flèche indique la direction centrifuge. Petite calotte centrifuge, sans amas périnucléaire.
d) Même traitement sur un œuf frère de *c*, mais récemment divisé en VIII. Petits amas centripètes et amas juxtanucléaire.

ténue, en se maintenant surtout à la surface des cellules enveloppantes (*e*, *f*). Cependant, l'amas enveloppé n'est pas sans prendre une certaine coloration rosée (*e*).

Comment va se comporter, après centrifugation, ce matériel riche en plasmalogène ? Va-t-il, comme le suggère sa solubilité dans les alcools, migrer au pôle centripète ? ou va-t-il accompagner en direction centrifuge les granulations auxquelles il paraît lié ? Avec la force utilisée (cf. p. 102) c'est, chez le Rat, cette seconde éventualité qui se réalise. Seuls, les œufs indivis forment une cape centripète, réduite et modérément positive (fig. 3, *a*). Aux autres stades, le plasmalogène intracellulaire suit la masse des granulations; toutefois, il tend à dessiner une lame surplombant l'amas granuleux sur sa face centripète. Le matériel localisé le long des cloisons profondes ne se déplace pas (fig. 3, b_1, b_2, *c*).

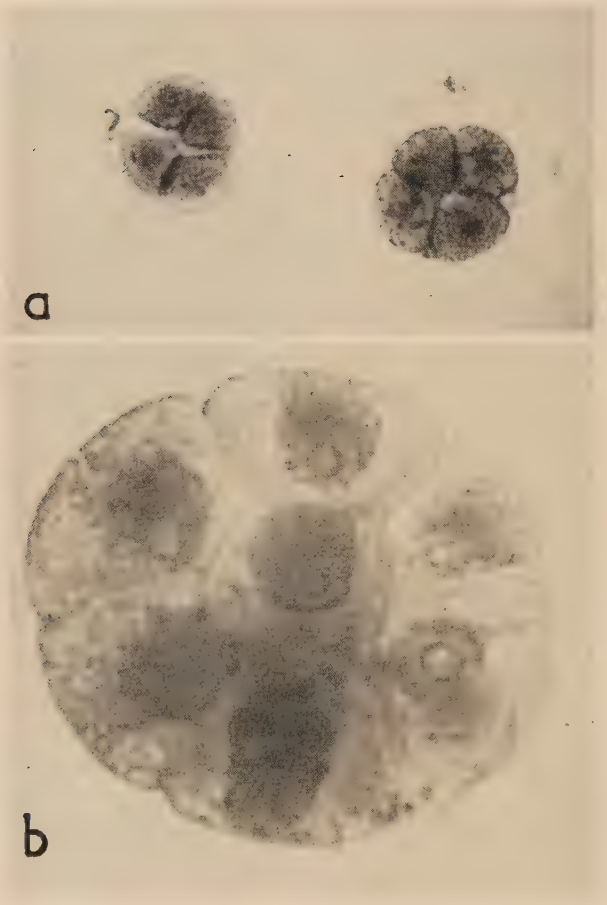


Fig. 5. R. 624 et 627. Réaction du plasmalogène *a*) dans deux œufs âgés de ± 58 h, avec coloration rouge surtout autour des noyaux et sur les surfaces de contact entre les blastomères; *b*) dans un œuf agé de ± 78 h, et divisé en VII blastomères, avec coloration rouge dans les amas juxtanucléaires de mitochondries, surtout dans les 3 gros blastomères à gauche et en bas (éléments qui vont envelopper). Photos Ektachrome retransposées en noir, *a* × 250, *b* × 570.

Considérons le *cas de la Souris*. On sait que, dans cette espèce, la métachromasie se manifeste d'une façon plus diffuse que chez le Rat. On ne trouve pas ces amas en essaims, si caractéristiques de cette dernière espèce. Il en va de même pour le plasmalogène. L'œuf prend plutôt une coloration rosée générale (fig. 4, *a*, *b*). Un examen attentif permet cependant de reconnaître une condensation juxtanucléaire. De même, la centrifugation (fig. 4, *c*, *d*) ne produit pas, à première vue, de stratification nette. Toutefois, au stade IV et VIII, chaque blastomère forme une petite cape centripète, et elle est positive au Schiff; néanmoins, la zone principale de plasmalogène se maintient à proximité du noyau.

Remarques sur l'organisation du cytoplasme ovulaire chez le Rat

Au cours des études antérieures, il n'avait pas été possible de percevoir, dans les œufs en segmentation, des différences permettant de distinguer la partie animale de

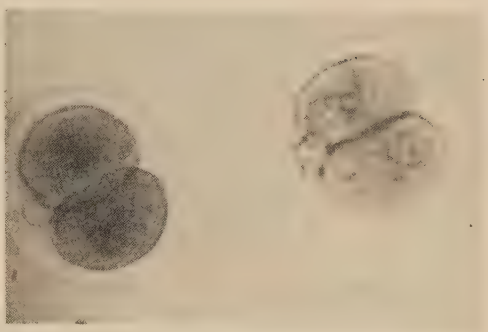


Fig. 6. Effet de l'âge sur la coloration *in vivo* au bleu de toluidine 10^{-5}. Les œufs des ♀ 679 et 680, respectivement du 3e et du 2e jour ont été placés dans des conditions identiques, puis photographiés côte à côte en Ektachrome; l'image a dû être retransposée en noir. A gauche, œuf du groupe âgé de ± 53 h, s'approchant de la nouvelle division : gros noyau avec 2 ou 3 nucléoles, métachromasie accentuée autour du noyau. A droite, œuf du groupe agé de ± 29 h, noyau pourvu de 6–8 nucléoles, métachromasie bien plus légère sur des petits groupes de granulations dispersées dans tout le cytoplasme.

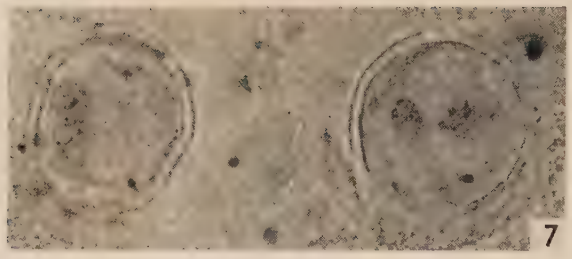
la végétative. Cette lacune a été comblée au cours des observations décrites ici. Aux stades IV et VIII, du type régulier, chaque blastomère se montre polarisé dans le sens An–Vg. On y reconnaît (fig. 2, b_2) au dessus du noyau, qui est décalé dans le sens animal, une zone de cytoplasme relativement pur, tandis qu'en dessous du noyau se trouvent l'essaim de mitochondries métachromatiques et son plasmalogène interstitiel, puis la zone lacunaire qui occupe toute la partie végétative. De plus, au stade VIII, les blastomères périphériques ont leur axe orienté obliquement de sorte que leurs parties animales entourent une dépression; le fond de celle-ci est occupée par la cellule centrale, parfois par les deux éléments qui, dans un certain mode de clivage, occupent le fond de la fossette.

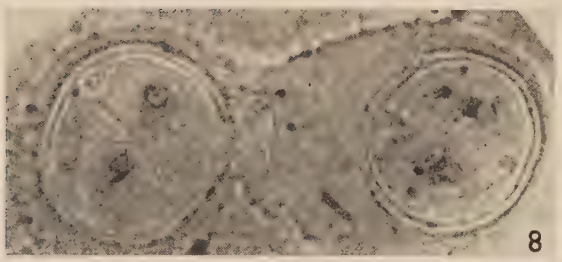
Ajoutons que la différence souvent soulignée entre les moitiés dorsale et ventrale de la placula dépend en grande partie de la proportion entre le cytoplasme dense et la portion lacunaire, plus abondante du côté ventral (fig. 2, *a*). On saisit ainsi, très simplement, la filiation des caractéristiques structurales aux stades successifs. Comme il a été montré précédemment ([3], p. 250, [5] p. 28), la symétrisation initiale de l'œuf dépend seulement du fait que la partie dense et basophile n'occupe pas exactement le pôle animal, mais est reportée d'un côté de l'axe An–Vg, tandis que la partie lacunaire, surtout végétative, est décalée en sens opposé. Compte tenu du remaniement imposé par les mitoses successives, l'organisation aux stades IV et VIII dérive de ces dispositions initiales.

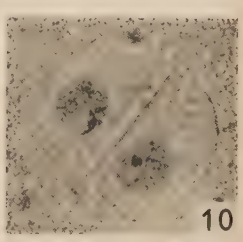
La réaction de Feulgen

Pour les raisons indiquées à propos de la partie technique de ces recherches, la réaction de Feulgen se borne à mettre en évidence le DNA des chromosomes, et son principal intérêt a été de permettre les évaluations photométriques qui ont été menées à bien avec J. Pasteels [9]. Seuls, certains aspects qualitatifs seront relevés ici.

Effets de la centrifugation sur la conformation des noyaux. —La réaction de Feulgen a déjà été décrite dans les noyaux d'œufs centrifugés [4] en insistant notamment sur l'étroite liaison entre les chromosomes et les nucléoles. Si l'on y est revenu, au moment où l'on explorait les possibilités de la fixation au formol calcique, c'était dans le but de rassembler les chromosomes en un amas favorable à la photométrie. Cet espoir a été déçu. En effet, tandis que les divers nucléoles fusionnent en un co-nucléole







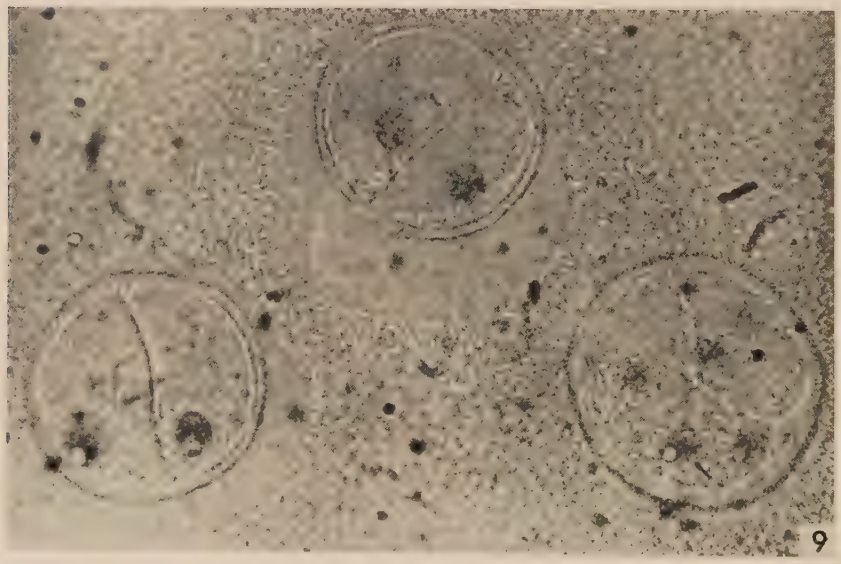


Fig. 7. R. 646 et 647 b. Deux spécimens d'un échantillon mixte, fixé au formol calcique, en comprenant des œufs centrifugés indivis, âgés de ± 12 h, et des œufs divisés en II, âgés de ± 34 h. A gauche, le seul des œufs indivis où une réaction positive réapparaisse dans les pronucléi (à gauche) : le pronucléus ♀ est au dessus, et plus positif que le pronucléus ♂, dont on ne voit que le gros nucléole. A droite, un œuf divisé en II, une ombre oblique indiquant la limite des blastomères;

géant, les chromosomes étirés par les corpuscules nucléolaires dessinent une sorte de chevelure flottant à l'arrière du co-nucléole. L'hétérogénéité du noyau est donc plutôt augmentée que réduite, ce qui nuit à la photométrie. Cependant, quelques constations sont à retenir :

Différences entre les noyaux du stade II et les pronucléi

R. 644 (1er jour) et R. 645 (2d jour) sont opérés simultanément dans l'après-midi et leurs complexes centrifugés pendant 7 + 45 + 7 min. Les œufs 645 a, stades II âgés de ± 36 h, ont des noyaux allongés avec gros co-nucléole, le nucléoplasme occupé par une trame de DNA, avec gradient vers le nucléole. Les œufs 644 a, stade I âgés de ± 12 h, ont des pronucléi ♀ petits, discrètement positifs, des pronucléi ♂ déjà énormes, sans réaction nettement perceptible. (Voir fig. 7, bien qu'elle concerne d'autres œufs.)

Différences entre les noyaux II jeunes et avancés

Les seconds complexes des mêmes animaux sont prélevés le lendemain matin et centrifugés pendant 7 + 65 + 7 min. On obtient ainsi 2 lots 644 b (stade II âgés de ± 30 h) et 645 b (stades II âgés de ± 54 h). Dans les premiers, le gradient de DNA est assez accentué, avec un revêtement violet coiffant le co-nucléole (comme dans la fig. 7, œuf de droite). Dans les seconds, les noyaux sont tout aussi étirés, mais le DNA est réparti sur une trame de chromosomes distincts, parallèles, renforcés par place (fig. 8). Visiblement, la structure nucléaire est modifiée, mais on n'a pas l'impression d'un enrichissement certain en DNA.

Enrichissement brusque des pronucléi vers la 10e ou 12e heure

R. 646 (1er jour). A 15 h et 17 h, prélèvement successif et centrifugation (7 + 65 + 7 min.) des deux complexes. On obtient ainsi 5 œufs d'âge présumé 10 h et 12 h. Parmi eux, 1 seul, du 2d lot, montre des pronucléi ayant une réaction perceptible (fig. 7, œuf à gauche), les autres sont encore complètement incolores. Si l'on rapproche ce résultat de celui fourni par R. 644 a, on est induit à penser que l'assimilation du DNA s'opère vers l'âge de 10 à 12 h et serait assez brusque.

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les deux noyaux sont allongés et le co-nucléole est refoulé du côté centrifuge; le nucléoplasme, faiblement hétérogène, est nettement plus positif que celui des pronucléi. (× 500.)

Fig. 8. R. 645 b. Deux âgés de ± 54 h, centrifugés, fixés au formol calcique et traités au Feulgen (× 500). Le sens de la centrifugation est indiqué par le volumineux co-nucléole. Auprès de celui-ci, groupe de chromosomes attirés vers le co-nucléole (surtout visibles dans le noyau inférieur de l'œuf gauche et dans le noyau supérieur de l'œuf droit).

Fig. 9. R. 648 a. Œufs centrifugés âgés d'environ 57 h, dont 2 stades II en imminence de division, et un stade IV récemment formé. Fixation au formol calcique et Feulgen (× 500). Dans chaque noyau, un gros nucléole indique le sens de la centrifugation. Les noyaux préprophasiques du stade II n'ont pas été étirés par le traitement, contrairement aux stades plus jeunes. Les jeunes noyaux filles sont également restés globuleux. Remarquer leur colorabilité réduite par rapport à ceux des stades II.

Fig. 10. R 675. Œuf normal, non centrifugé, recueilli ± 53 h après une copulation diurne, fixé à l'alcool froid, traité au Feulgen (× 500). La prophase est imminente. Dans le noyau gauche, le gros nucléole n'est plus recouvert de la pellicule de DNA que dans sa moitié droite : il se déshabille littéralement.

Evolution dans la déformabilité des noyaux

R. 648 (3e jour), opérée successivement, à 3 h d'intervalle, de manière à centrifuger chaque complexe pendant 7 + 70 + 7 min. On obtient a) 2 œufs en II et 2 en IV, âge présumé 57 h; b) 1 œuf en III et 2 en IV, âge présumé 60 h. On peut donc observer les effets de la centrifugation sur des noyaux prophasiques d'une part, post-télophasiques d'autre part. Pour les 1ers, l'aspect de la chromatine est, comme à 54 h, une trame de filaments parallèles, bien individualisés. Le fait remarquable est qu'à ce stade il ne se produit plus d'élongation des noyaux. Il en est d'ailleurs de même pour les jeunes noyaux du stade IV qui voisinent avec les stades II avancés (fig. 9).

Appréciation de la richesse probable en DNA

L'impression purement visuelle se résumait dans : l'extrême pâleur des jeunes pronucléi; leur enrichissement brusque vers la 10e–12e h, mieux perceptible dans le pronucléus ♀ que dans le ♂, à cause du gonflement marqué de celui-ci; des valeurs peu différentes pour les noyaux II qu'ils soient observés à 30 h (soit 5 ou 6 h après la 1re division) à 54 ou à 57 h; des valeurs nettement inférieures, probablement moitié moindres, pour les jeunes noyaux IV. Le recours aux mesures photométriques s'imposait donc, à condition d'améliorer encore la technique : ce qu'a permis, à ce moment, l'emploi de l'alcool froid.

La fixation à l'alcool froid.—Ce procédé a donné, sur les noyaux normaux, des images suffisamment définies et d'une dispersion assez régulière que pour renoncer à la centrifugation. Les aspects qui ont été ainsi mis en évidence confirment nos observations antérieures [6], notamment quant à la genèse des nucléoles, à leur évolution au cours de chacun des cycles, aux émissions de vésicules nucléolaires ou de bulles de nucléoplasme, à la liaison entre chromosomes et nucléoles pendant les intercinèses. Les détails suivants méritent d'être consignés ici.

Libération finale des nucléoles

Il a été signalé ([6], p. 1357) qu'à la prophase le gros nucléole[1] qui subsiste encore se détache des chromosomes en voie de rétraction. Cette fois, dans des œufs du stade II âgés de ± 53 h (R. 675), cette libération elle-même a été surprise (fig. 10) : on a trouvé certains nucléoles revêtus de DNA uniquement sur la moitié tournée vers le chromosome correspondant. Le mécanisme de libération est donc bien celui qui avait été supposé : la pellicule de DNA qui jusque là revêtait entièrement le nucléole s'amincit jusqu'à se rompre en un point, et la couche enveloppante se rétracte en dénudant la vésicule.

Les chromosomes métaphasiques et les vésicules métachromatiques des mitoses

Assez d'images de chromosomes en métaphase et en anaphase des premières mitoses ont été revues que pour les distinguer des vésicules décelées au même stade, dans les œufs fixés au S.P.W.M.après coloration vitale au Bleu de toluidine ([6], p. 1345). Toutefois, jusqu'ici, les vésicules décrites par Izquierdo n'ont pas été observées en même temps que les chromosomes. S'il y a vraiment coexistence des deux structures, l'origine des vésicules serait à rechercher.

[1] Il y en a parfois deux et même trois.

Etat du DNA dans les très jeunes pronucléi

Un effort a été fait pour retrouver, par cette nouvelle technique, les aspects spéciaux signalés par K. S. Ludwig : très jeunes pronucléi négatifs au Feulgen mais positifs au Schiff. Cet essai est de nouveau resté infructueux. Il ne faudrait cependant pas conclure à une erreur. Même si la fixation aux seules vapeurs d'alcool est devenue sujette à caution pour ces stades indivis, les préparations faites par notre ancien collaborateur n'en subsistent pas moins. Je n'ai d'ailleurs pas retrouvé non plus l'envahissement de la pièce intermédiaire par le DNA ([6], fig. 11) détail dont la réalité ne fait aucun doute. Il y a, dans cette période initiale, des processus singulièrement fugaces.

DISCUSSION

L'enquête dont il était ici rendu compte avait pour objectif final la détermination photométrique des valeurs relatives du DNA dans les noyaux de segmentation. Ce résultat a été atteint au prix de tâtonnements dont beaucoup n'ont eu qu'un résultat négatif ou ne se justifient que par le souci de lever des objections possibles, tandis que certaines données sont un apport appréciable à notre connaissance de l'œuf chez les deux Rongeurs utilisés.

Il est tout d'abord acquis que dans ces œufs, pas plus que dans tant d'autres examinés à ce point de vue, il n'existe dans le cytoplasme ni DNA ni complexe du type « acide thymique » que le réactif de Schiff puisse mettre en évidence. Ceci ne vaut évidemment que dans la limite de la méthode utilisée — soulignons qu'il s'agit toujours de montage *in toto* — et n'exclut pas la présence, à certains stades, de matériaux pouvant servir de précurseurs immédiats au DNA nucléaire; une méthode biologique telle celle employée par Hoff-Jørgensen et Zeuthen [10] serait à envisager à ce propos, si le matériel pouvait être obtenu en quantité suffisante.

Le plasmalogène vrai est un constituant banal des cellules, et Lison ([14], p. 383) rapporte qu'il pourrait être considéré, d'après Pischinger, comme correspondant à des acétalphosphatides probablement liés aux protéines en des complexes lipoprotéiques.

L'étude faite ici s'inspire plus d'un esprit morphologique que biochimique, et l'auteur est conscient d'avoir laissé certains aspects inexplorés. Il aperçoit la possibilité d'une nouvelle recherche qui, bénéficiant de l'expérience acquise, repartirait d'œufs fixés au formol calcique d'une part, à l'alcool froid d'autre part, pour leur appliquer une hydrolyse dans HCl *N*/10 à la température ordinaire et effectuer au moins quelques-unes des réactions discriminatives suggérées par Danielli [4].

Tel que le plasmalogène nous est jusqu'à présent apparu, il constitue un complément estimable à notre représentation de l'œuf des Rongeurs. Ce qui

marque son intérêt, c'est le fait de ne pas avoir la valeur d'une réserve quelconque, qui s'épuiserait graduellement pendant la phase de vie libre de l'œuf, mais d'être au contraire formé progressivement en liaison avec les cycles de vie cellulaire. Cet intérêt s'accroît par le parallélisme topographique et quantitatif qui s'affirme avec les granulations spéciales que révèle la métachromasie *in vivo* au bleu de toluidine. Il est acquis que ces granulations sont des mitochondries (test de Baker [6]) et que leurs propriétés tinctoriales tiennent à la présence, à leur niveau, de mucopolysaccharides. Cette dernière notion, d'abord fondée sur des arguments quelque peu indirects, vient d'être consolidée par une démonstration cytochimique directe [16]. Le parallélisme quantitatif et topographique se dégage 1) de l'étude des œufs normaux du Rat aux divers stades, y compris l'atténuation caractéristique lors de la mitose, 2) des effets de la centrifugation, 3) de l'examen des œufs de Souris considérés dans les mêmes conditions. La comparaison des deux espèces est tout à fait significative. Chez la Souris, les mitochondries sont plus fines, d'une densité peu différente de celle du cytoplasme; la force centrifuge employée ne les déplace guère, mais une partie du plasmalogène peut se dégager en direction centripète. Chez le Rat, les mitochondries sont plus grosses et relativement plus lourdes, de sorte qu'elles entraînent le plasmalogène interstitiel, jusqu'au moment où l'accumulation des grains dégage plus ou moins ce composant plus léger.

On a été particulièrement attentif à préciser si le plasmalogène était localisé *sur* ou *entre* les granulations, problème déjà rencontré à propos de la fixation de la métachromasie. Sans être trop exclusif, on peut affirmer que le plasmalogène est surtout interstitiel, tandis que les mucopolysaccharides sont surtout liés aux granules mitochondriaux. On s'est aussi demandé si la réaction de Schiff n'intéressait pas les noyaux, ce que semblaient indiquer les premières préparations. Sans doute s'agissait-il de surcoloration secondaire. Par la suite, les noyaux ont pu être mis nettement hors cause.

Ainsi se dessinent peu à peu des relations cytologiques dont les mailles se resserrent autour de processus biochimiques qui restent eux-mêmes en partie énigmatiques. Il a été montré [6] que la production de mucopolysaccharides au niveau de certains amas mitochondriaux dépend de l'inoculation, dans ces derniers, de vésicules nucléolaires et d'émissions nucléaires analogues. Il apparaît maintenant qu'en ces mêmes endroits l'hyaloplasme interstitiel se charge de ces complexes phosphatidiques qualifiés de plasmalogène. Ces deux conséquences des instillations nucléaires sont visiblement liées. De plus, sans que la raison en apparaisse clairement, elles n'intéressent pas seulement la région périnucléaire, mais aussi le cortex des

cellules, plus particulièrement les zones profondes de contact entre les blastomères. Plus significatif est encore le fait que ces élaborations se poursuivent durant toute l'intercinèse *jusqu'à un enrichissement maximum*. Dès 1951, l'affaiblissement de la métachromasie *in vivo* avait été remarqué lors de la mitose. Ce n'est là, comme on le discerne à présent, que la contrepartie de cet enrichissement progressif durant l'intercinèse, lequel constitue le processus primordial. En effet, la conclusion majeure des mesures photométriques [9] a été que l'assimilation du DNA s'opère immédiatement après chaque segmentation, approximativement pendant le 1er quart de la nouvelle période intercinétique. La mitose suivante ne dépend pas, contrairement à ce que j'ai pu admettre ([6] p. 1371), de la réalisation de cette valeur nucléique, condition peut-être nécessaire[1] mais sûrement pas suffisante. *La nouvelle caryocinèse est subordonnée à l'élaboration de matériaux qui sont les précurseurs, plus ou moins immédiats, du DNA nécessaire aux jeunes noyaux-filles*, et dont l'accumulation, au delà d'un certain seuil, déclanche la prophase. De ce point de vue, l'enrichissement graduel en mucopolysaccharides et en plasmalogène présente un intérêt évident, surtout s'il est causé par des produits d'origine chromosomiale, *via* l'activité nucléolaire. En un mot, on voit se préciser la notion des *deux cycles corrélatifs* dégagée en 1954 ([6], 1368 et suiv.).

Certes, nous ne voyons pas, actuellement, les transformations biochimiques sous-jacentes à ces étapes du cycle cellulaire : ni entre le DNA et les protides des chromosomes et les constituants des nucléoles, protides, phospholipides, RNA; ni entre ceux-ci et les mucopolysaccharides surchargeant les mitochondries dans une ambiance de plasmalogène, au sein d'un cytoplasme tout imprégné de polysaccharides, particulièrement de glycogène; moins encore entre ces complexes lentement accumulés et les éléments divers, sucres, bases organiques, molécules phosphorées, peut-être des nucléotides tout constitués, qui vont permettre aux noyaux-filles de réparer leur déficience en DNA. C'est que nous n'avons encore que quelques repères fournis par les métabolites qu'il a été possible de détecter dans ces œufs et leurs blastomères, et moins encore d'informations directes quant aux enzymes actifs dans ces transformations. Nous savons tout juste que certaines catégories de phosphatases alcalines ne sont pas en cause, tandis que la cytochrome-oxydase des mitochondries est sûrement active [1], comme, sans doute, bien d'autres de leurs ferments.

C'est là, semble-t-il, la conclusion la plus importante de cette investigation. Elle a fourni, en outre, un complément d'information sur l'organisation

[1] Voir, dans l'étude photométrique, la discussion de certaines anomalies.

générale du cytoplasme. Reconnaître ainsi que les blastomères possèdent une polarité structurale selon l'axe animal-végétatif ne manque pas d'intérêt pour l'Embryologie comparée. C'est, par ailleurs, une justification complémentaire, et une meilleure élucidation, de la présence, dans ce système germinal, de différences dorso-ventrales qui lui confèrent un plan de symétrie bilatérale. Il ne s'agit toutefois pas de deux ordres de propriétés distinctes, mais simplement d'un décalage par rapport à l'axe fondamental des parties denses et lacunaires, les premières à prédominance animale, les secondes à prédominance végétative. Par surplus, il pourra éventuellement y avoir quelque importance à pouvoir orienter les placules des stades VIII à XII grâce à leur fossette animale.

Enfin, si classique que soit la combinaison de l'hydrolyse avec l'emploi du Schiff, il n'est pas inutile d'être assuré que ce traitement n'a d'autre vertu que le démasquage du DNA chromosomial. En particulier, il est sans action sur la détection du plasmalogène (après action du sublimé) et, par ailleurs, la délipidation préalable reste sans retentissement sur le Feulgen.

A propos des quelques observations qualitatives qu'ont permis ces préparations, il y a peu de commentaires à ajouter. L'allongement des noyaux dans les œufs centrifugés aux stades I et II reste un phénomène remarquable, signe de la présence d'un constituant léger dans le nucléoplasme. Ce matériel a pu être coloré par la pyronine [2] .Que cet allongement cesse de se produire à la fin du stade II et ne se manifeste plus aux stades ultérieurs pourrait dépendre de la disparition de ce constituant du nucléoplasme, mais pourrait aussi provenir d'un changement dans le cytoplasme.

Les remarques faites à propos des nucléoles, de leur déhiscence à l'égard des chromosomes qui les ont sécrétés, de leurs relations possibles avec les vésicules métachromatiques aperçues pendant la mitose doivent être considérées surtout comme des jalons appelant de nouvelles recherches.

RÉSUMÉ

L'action du réactif de Schiff a été étudiée sur les œufs en segmentation du Rat et de la Souris.

Les conditions les plus favorables pour la mise en évidence du plasmalogène vrai et du DNA ont été méthodiquement définies.

Le plasmalogène présente une localisation et une évolution identique à celle des groupes mitochondriaux chargés de mucopolysaccharides, à cela près qu'il est diffus dans l'hyaloplasme interstitiel. Un ensemble d'indications plaident en faveur de l'idée que ces constituants sont étroitement liés

au matériel précurseur du DNA et responsable du déclenchement de la division suivante.

Le comportement général des noyaux dans les œufs centrifugés et la disposition de leurs chromosomes ont été décrits. On a de plus observé dans les œufs normaux la manière dont les nucléoles, au seuil de la prophase, se libèrent de leur revêtement de DNA.

Une méthode utilisable pour la détermination photométrique de la teneur relative des noyaux de segmentation en DNA a été mise au point.

SUMMARY

The effects of Schiff's reagent on eggs of the rat and the mouse have been studied during early and late cleavage.

The optimum conditions for the cytochemical detection of true plasmalogene and of DNA have been investigated.

The localization and accumulation of the plasmalogene are identical to the distribution of the mitochondrial groups loaded with mucopolysaccharides, except that the plasmalogene is bound to the interstitial hyaloplasm, and not to the mitochondria.

The available information points towards the idea that both these materials are intimately linked with some precursor of DNA, and that they are related to the onset of the new division.

The effects of centrifugation on the egg nuclei and on the distribution of their chromosomes have also been described. In normal eggs, the process by which nucleoli free themselves of their DNA coat, just before prophase, could be observed.

An appropriate method has been established for the cyto-photometric determination of the relative DNA contents of egg nuclei; the results of the measurements have been published with J. Pasteels [9].

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CYTOCHEMICAL AND AUTORADIOGRAPHIC OBSERVATIONS ON NUCLEAR RIBONUCLEIC ACID IN *AMOEBA PROTEUS*

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The nuclear control of synthetic processes of the cell has been proposed repeatedly. In particular a mechanism has been sought whereby genes can transmit their specificity to a cytoplasmic component. Nuclear ribonucleic acid (RNA) is a good candidate for the role of primary transmitting agent (cf. [7, 15, 18]). Although there is general agreement on the greater metabolic activity of nuclear RNA relative to its cytoplasmic counterpart (see, for instance, [1, 9, 13]), the interpretation of this fact in terms of the biosynthetic relationship between nuclear and cytoplasmic RNA is not agreed upon (cf. [14, 16]). An examination of the history of nuclear RNA in intact cells, using reliable cytochemical procedures to demonstrate gross changes in amount and isotopic labelling techniques for the detection of more subtle changes and turnover rates, may serve to support or qualify the aforementioned candidacy of this substance as a gene-cytoplasm link.

The amoeba, whose nucleus contains histochemically detectable RNA [3, 19] should be a favorable organism for such an investigation. In this preliminary note we wish to report on the localization of nuclear RNA in *Amoeba proteus*, its behavior during division, and in the nuclei of isotopically labelled fed and starving cells.

Amoebae were cultivated in modified Hahnert's medium at 18° C and fed with washed *Tetrahymena pyriformis*, as described by Prescott [17]. The amoebae were squashed on slides in 45 per cent acetic acid (with and without 2 per cent lanthanum nitrate), frozen over solid CO_2 to facilitate cover slip removal, immersed in acetic acid : ethanol (1:3), ethanol, and finally air dried. The fixed amoebae were subsequently treated with (a) ribonuclease (0.02 per cent in distilled water, pH adjusted to 7.0, at 25° C for four to six hours), (b) distilled water under identical pH, temperature,

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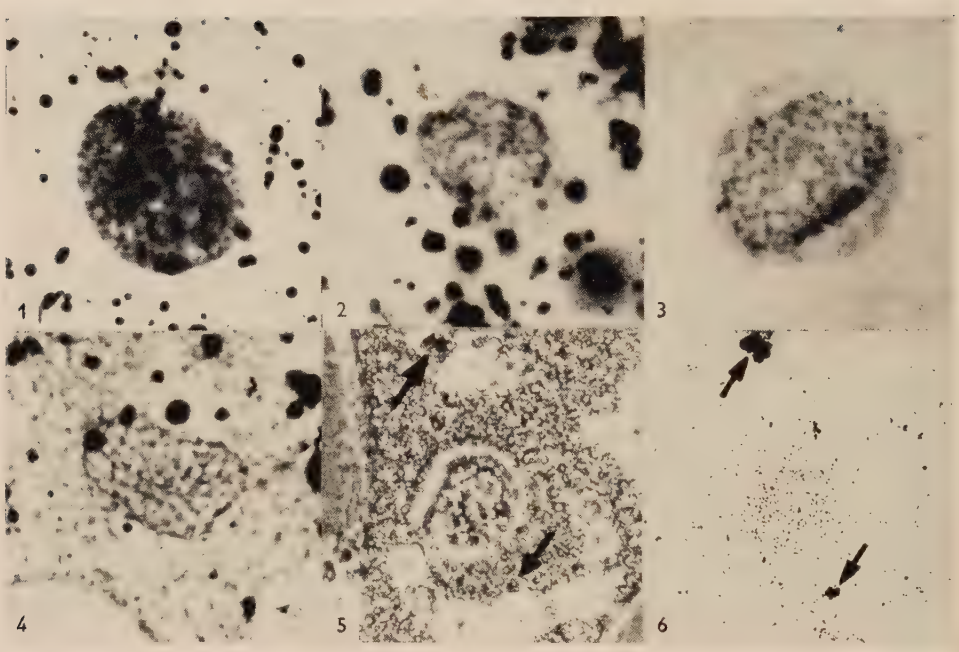
and time conditions for ribonuclease control, (c) perchloric acid (10 per cent in distilled water at 4 to 6° C for 16 to 24 hours), and (d) cold, pH unadjusted distilled water as perchloric acid control. Staining was performed with (a) azure B at pH 4.0 [10] and (b) gallocyanin-chromalum at pH 1.8 [8]. Autoradiography was carried out by the stripping film technique of Doniach and Pelc [6], employing the autoradiographic stripping emulsion of Kodak Ltd. The emulsion-covered slides underwent a four week exposure period prior to photographic development.

The stained slides showed that the cytochemically demonstrable nuclear RNA is homogeneously distributed over the whole squashed nucleus in the form of granules (Fig. 1). These granules correspond to the "peripheral granules" described from observations on sectioned material by Bogdanowicz [2], Dawson *et al.* [5], and Liesche [12]. It is to be noted that the first of these authors called attention to the bright staining of the granules with pyronin. The addition of 2 per cent lanthanum nitrate to the 45 per cent acetic acid fixative resulted in no noticeable difference in the staining. (In view of the fact that the addition of lanthanum did, however, produce undesirable artefacts in the stripping emulsion, it was eliminated from the routine procedure.) Ribonuclease digested or perchloric acid extracted material revealed a much more faintly stained reticular substance, occupying the non-peripheral portion of the nucleus (Figs. 2 and 3). This latter affinity for the dye could be abolished by pre-treatment with hot trichloracetic acid (5 per cent at 90° C for 20 minutes) and significantly reduced by desoxyribonuclease digestion. The fact that a very faint but morphologically similar picture was obtained after Feulgen treatment suggests that this reticular substance contains DNA (cf. [2]). Visual inspection indicates that the RNA content of the *A. proteus* nucleus is considerably higher than its DNA content.

In amoebae fixed at different times after cytokinesis it was observed that the staining of nuclei of newly separated cells indicated little or no RNA-containing material (Fig. 4), so that the undigested and unextracted nuclei at this stage bear a strong resemblance to the nuclei of predivision cells extracted with ribonuclease or perchloric acid. From one or two hours after the separation of daughter cells cytochemically detectable RNA reappears in the nuclei; by four hours after division the nuclear picture is indistinguishable from that of pre-division controls. ("Predivision" cells were randomly collected amoebae which showed no morphological indications of approaching division.) Localization of RNA in the peripheral granules and its reduction after division agrees with what has already been reported on the behavior of these granules [4, 5, 12].

Simultaneously with the cytochemical findings briefly outlined above we



Figs. 1–4. *Amoeba proteus* stained with gallocyanin-chromalum. Magnification: ×550.
Fig. 1. Nucleus of non-dividing amoeba, treated with distilled water for 4 hours (ribonuclease control). Note staining of granules in the nucleus.
Fig. 2. Nucleus of non-dividing amoeba after 4 hours of ribonuclease digestion.
Fig. 3. Nucleus of non-dividing amoeba after 24 hours of cold 10 per cent perchloric acid extraction. Notice the relatively faint staining of the nuclei in Figs. 2 and 3; only DNA is stained
Fig. 4. Nucleus of post-division amoeba, undigested and unextracted. Note similarity in staining to Figs. 2 and 3.
Fig. 5. Phase contrast photograph of unstained nucleus of non-dividing amoeba, fixed after 4 hours in a 1μ C/ml adenine-8-C^{14} solution.
Fig. 6. Non-phase photograph of developed stripping film over amoeba nucleus shown in Fig. 5. The field covered is the same. Arrows on Figs. 5 and 6 point to corresponding landmarks which can be used to locate nuclear area in Fig. 6. Magnification of Figs. 5 and 6: ×283.

found that nuclear RNA in *A. proteus* is readily labelled with adenine-8-C^{14} (cf. [1, 11]), administered by placing amoebae in normal medium [17] at 25° C to which approximately one microcurie of the isotope per milliliter had been added. Autoradiographic detection of the isotope in the nucleus was possible after two hours or more of incubation of the amoebae in the labelled medium (Figs. 5 and 6). In fixed amoebae digested with ribonuclease or extracted with perchloric acid no nuclear autoradiograph could be observed, indicating that under these conditions no appreciable incorporation of the isotope into DNA occurred.

Of several tracer experiments initiated as a result of these developments

only the first has progressed to the stage where a preliminary interpretation is possible. A large number of amoebae was incubated in adenine-8-C^{14}-enriched medium for 17 hours. The cells were then washed in unlabelled medium and transferred to fresh, unlabelled medium in two culture vessels. One culture was subsequently fed with *Tetrahymena* at regular intervals while the other culture received no food for the course of the experiment. Fixations of amoebae were made from both cultures over a period of ten days.

Qualitative scanning of the photographically developed preparations indicates a sharp difference in isotopically labelled RNA in the nuclei of the starving and the fed cells as a function of time elapsed after labelling: the starving amoebae show nuclear autoradiographs of gradually diminishing intensity over the entire range of ten days; the fed cells, on the other hand, show the total absence of nuclear label after three to six days. (The RNA localization of the label is indicated by its absence in the nuclei of cells fixed immediately after the 17-hour incorporation period and digested with ribonuclease.)

How much of the loss of RNA label from the nuclei of the fed group of amoebae is due to the cytochemically demonstrated loss at division and how much must be ascribed to turnover or a more gradual loss cannot be ascertained until more data become available. That more than the division loss is involved seems to us likely in view of the gradual decrease in label in the nuclei of the starving amoebae. (There is, however, some evidence that nuclear RNA decreases after fasting in amoebae [3] and liver cells [13]; in the latter case it has been shown that the level of energy intake influences the nuclear RNA turnover [20]). At present we should like to restrict ourselves to pointing out that an extensive loss of cytochemically demonstrable nuclear RNA occurs at division in *A. proteus*, and that fed and fasting amoebae show a striking difference in the behavior of adenine-8-C^{14} incorporated into nuclear RNA. Experiments to elucidate further the history of nuclear RNA label are in progress and will be published together with the detailed analysis of the data summarized in this report.

We would like to express our gratitude to Professor Daniel Mazia for his continued interest and valuable suggestions throughout the course of this work, to Dr. E. L. Bennett for his generosity in providing some of the labelled adenine used, and to Dr. D. M. Prescott for his initial help in working out the technique for the administration of the isotope to the amoebae.

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UNTERSUCHUNGEN ÜBER DEN EINFLUSS VON RÖNTGENSTRAHLEN AUF DIE DESOXYRIBONUCLEINSÄURE IM WALKER-CARCINOM DER RATTE

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Seit den Untersuchungen von Eulers und von Hevesys [9, 10] ist es bekannt, dass ionisierende Strahlen die Neubildung der Desoxyribonucleinsäure (DNS) im bestrahlten Gewebe hemmen. Bezüglich des Mechanismus dieser Hemmwirkung sind noch zahlreiche Fragen offen. Mit den nachstehend geschilderten Versuchen sollte geklärt werden, ob die Synthesehemmung die DNS in einem Rattentumor gleichmässig trifft oder ob innerhalb der DNS des Gewebes eine unterschiedliche Strahlenempfindlichkeit bestehen kann. Anlass zu diesen Untersuchungen waren die Beobachtungen Bendichs [4, 5, 6], dass sich aus einem Gewebe 2 DNS-Fraktionen isolieren lassen, die sich stoffwechselmässig verschiedenartig verhalten können. Die Trennungsmöglichkeit dieser beiden DNS-Fraktionen beruht darauf, dass die eine (DNS_1) in 0,87%iger Kochsalzlösung ausfällt, während die andere (DNS_2) in Lösung bleibt.

Bei den vorliegenden Untersuchungen wurde die von Bendich angegebene Methode zur Isolierung der Nucleinsäuren am Walker-Carcinom der Ratte angewandt, um den Einbau von Radiophosphor in die beiden DNS-Fraktionen im unbestrahlten und im röntgenbestrahlten Tumor zu bestimmen.

METHODIK

Versuchstiere waren 42 Wistar-Ratten von 170 bis 210 g mit subcutan transplantiertem Walker-Carcinom.[1] P^{32} wurde als Na_2HPO_4 intraperitoneal injiziert (Dosis 2 μC/g Körpergewicht), bei den bestrahlten Tieren jeweils unmittelbar nach der Bestrahlung des aus einem Bleigehäuse herausgelagerten Tumors (5000 r bei 180 kV, 10 mA, 0,5 mm Cu gefiltert; Dosisleistung 290 r/min). Die Zeitspanne zwischen der

[1] Das Walker-Carcinom stellte uns freundlicherweise Herr Prof. Dr. Hoepke, Direktor des Anatomischen Instituts der Universität Heidelberg, zur Verfügung.

Injektion des Radiophosphor und der Tötung der Tiere betrug jeweils 2, 12 und 24 Stunden.

Nach Entfernen nekrotischen Materials wurde das Tumorgewebe in Alkohol, der mit Trockeneis (CO_2) versetzt war, homogenisiert und nach Spülen mit Alkohol, Alkohol-Äther und Äther im Exsiccator getrocknet. Das Gewebspulver wurde nun in dem 20fachen Volumen 10%iger NaCl-Lösung aufgeschwemmt und 6 Stunden lang bei einer Temperatur von 85° C gerührt. Aus dem abfiltrierten Kochsalzextrakt wurden die Nucleinsäuren mit dem 3fachen Volumen Alkohol gefällt (in der Kälte), mit Alkohol, Alkohol-Äther und Äther gespült und getrocknet. Die so isolierten Natriumsalze der Nucleinsäuren wurden noch einmal in 5%iger NaCl-Lösung (10faches Volumen) aufgeschwemmt, 15 min bei 85° C gerührt, abfiltriert, erneut mit Alkohol gefällt und nach Spülen mit Alkohol, Alkohol-Äther und Äther getrocknet. Die auf diese Weise gewonnenen Nucleinsäuren gaben keine positive Biuretprobe mehr.

Je 150 mg der isolierten Nucleinsäuren wurden nun in 15 ml 0,87%iger NaCl-Lösung aufgeschwemmt, 20 Stunden lang bei 3 bis 5° C gerührt und dann 1 Stunde lang zentrifugiert. Das sich absetzende Sediment bildete die DNS_1, während der Überstand DNS_2 und Ribonucleinsäure (RNS) enthielt. Die isolierte DNS_1 wurde noch zweimal mit 0,87%iger Kochsalzlösung gespült und dann nach weiterem Spülen mit Alkohol, Alkohol-Äther und Äther getrocknet. Aus der 0,87%igen NaCl-Lösung (Überstand + Spülflüssigkeit) wurden die in Lösung gebliebenen Nucleinsäuren wieder mit dem 3fachen Volumen Alkohol gefällt, abzentrifugiert und mit Alkohol-Äther und Äther getrocknet.

Die Trennung von DNS_2 und RNS erfolgte nach dem Prinzip der Schmidt-Thannhauser-Methode [25]. Die gemischten Nucleinsäuren wurden 10 Stunden mit 0,3 n NaOH im Wasserbad bei 37° C exponiert. Nach Abkühlen (Eisbad) und Neutralisieren mit 6 n HCl wurde so viel 10 %ige Trichloressigsäure (TCS) zugegeben, dass die Endkonzentration für die TCS 5 % betrug. Auf diese Weise kam es zur Fällung der DNS_2, während die hydrolysierte RNS in Lösung blieb. Nach Abzentrifugieren der DNS_2 wurde diese noch dreimal mit 5%iger TCS gespült.

Es erwies sich als nötig, auch die isolierte DNS_1 in gleicher Weise nach dem Schmidt-Thannhauser-Prinzip zu behandeln, um sie in ausreichender radiochemischer Reinheit zu gewinnen. Beide DNS-Fraktionen wurden dann anschliessend noch je dreimal wieder in 0,3 n NaOH gelöst, nach Neutralisieren mit TCS gefällt und weiter mit 5%iger TCS gespült. Vorversuche hatten gezeigt, dass die Wiederholung der Säurefällung — vor allem bei den 2 Stunden-Versuchen — zunächst stets zu einer Senkung der spezifischen Aktivität der isolierten DNS führte. Nach der zweimal wiederholten Fällung wurde die spezifische Aktivität durch weitere Fällungen jedoch nicht mehr verändert. Die Säurefällung der isolierten DNS-Fraktionen wurde daher jeweils viermal durchgeführt, um nicht durch radioaktive Verunreinigungen zu hohe Werte für die spezifische Aktivität zu erhalten.

Um die spezifische Aktivität der isolierten DNS-Fraktionen zu ermitteln, wurden aliquote Mengen zur Zählrohrmessung und zur colorimetrischen Phosphat-Analyse nach Gomori [11] verwandt. Zur Kontrolle der Analysewerte wurde ergänzend zum Phosphat der Kohlenhydratanteil der DNS mit Hilfe der Diphenylamin-Reaktion nach Dische [8] bestimmt.

Um das Mengenverhältnis DNS_1/DNS_2 im Tumor zu erfassen, wurden zusätzlich

quantitativ-chemische Untersuchungen ohne Radiophosphor durchgeführt. Bei diesen erfolgte die Bestimmung der DNS einmal mittels der Diphenylamin-Reaktion, und zwar für die DNS_2 ohne vorherige Trennung von der RNS. Zur Ermittlung des DNS-Phosphats wurden die beiden isolierten Nucleinsäure-Fraktionen nach dem Schmidt-Thannhauser-Verfahren behandelt, jedoch ohne die oben beschriebene Wiederholung der Säurefällung.

ERGEBNISSE

Im unbestrahlten Tumor zeigten DNS_1 und DNS_2 innerhalb des untersuchten Intervalls von 24 Stunden keine Unterschiede bezüglich des Einbaus radioaktiven Phosphats; das Verhältnis der spezifischen Aktivitäten DNS_1/DNS_2 betrug nach 2, 12 und 24 Stunden stets 1. Im bestrahlten Tumor fand sich dagegen nach 2 Stunden nicht nur eine wesentlich geringere P^{32}-Aufnahme, sondern zugleich war das Verhältnis der spezifischen Aktivitäten DNS_1/DNS_2 grösser geworden (Tab. I). Es geht daraus hervor, dass die Neubildung der DNS_2 offenbar stärker gehemmt wurde als die der DNS_1 [3]. Nach 12 Stunden war das Verhältnis der spezifischen Aktivitäten als Folge der Strahleneinwirkung immer noch etwas grösser als 1, nach 24 Stunden betrug es 1,0 wie im unbestrahlten Tumor. Der zeitliche Verlauf der Aufnahme des Radiophosphor (Abb. 1) lässt erkennen, dass mit der „Erholung" des DNS-Umsatzes auch das unterschiedliche Verhalten der beiden DNS-Fraktionen verschwindet.

Bei den geschilderten Untersuchungen stellte sich ferner heraus, dass durch die Röntgenstrahleneinwirkung auch das Mengenverhältnis von DNS_1 und DNS_2 im Tumor verändert wird. In einer besonderen Versuchsreihe wurde dieser Frage nachgegangen. Dabei zeigte es sich, dass als Folge der Bestrahlung der Anteil der DNS_1 an der Gesamt-DNS erheblich abnimmt (Tab. II). Dieser Befund ergab sich in gleicher Weise bei DNS-Phosphat-Bestimmungen

TABELLE I

Verhältnis der spezifischen Aktivitäten von DNS_1/DNS_2 (mit Standardabweichungen) im Walker-Carcinom der Ratte 2, 12 und 24 Stunden nach Verabfolgung radioaktiven Phosphats.

	Zeit nach Injektion des Radiophosphor	Verhältnis der spezifischen Aktivitäten DNS_1/DNS_2	
Bestrahlter Tumor	2 Stunden	$1{,}23 \pm 0{,}05$	($P<0{,}01$)
	12 „	$1{,}12 \pm 0{,}04$	($P<0{,}05$)
	24 „	$1{,}01 \pm 0{,}04$	
Unbestrahlter Tumor	2, 12, 24 Stunden	$1{,}02 \pm 0{,}04$	

wie bei Anwendung der Diphenylamin-Reaktion nach Dische. Obwohl beim Walker-Carcinom die Werte für das Mengenverhältnis DNS_1/DNS_2 relativ stark streuen, steht die Beeinflussung durch die Röntgenbestrahlung ausser Zweifel.

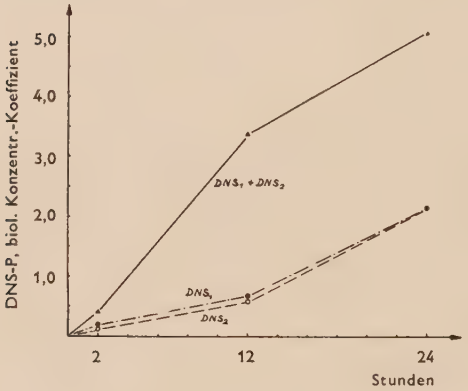

Abb. 1: Zeitlicher Verlauf der Aufnahme radioaktiven Phosphats in die DNS des Walker-Carcinoms der Ratte. Im unbestrahlten Tumor haben beide DNS-Fraktionen jeweils die gleiche spezifische Aktivität (▲); als Folge der Röntgenbestrahlung wird der Einbau des Radiophosphor gehemmt, und zwar bei der DNS_2 (○) mehr als bei der DNS_1 (●). — Die Radiophosphoraufnahme durch die DNS ist entsprechend den Vorschlägen von Schulman und Falkenheim [26] angegeben durch den „biologischen Konzentrationskoeffizienten" [12].

$$\left(\frac{\text{Zählrohrimpulse/min/milli-mol DNS-P}}{\text{Zählrohrimpulse/min des injizierten } P^{32} \text{ pro g Körpergew.}} \times 100\right)$$

TABELLE II

Mengenverhältnis DNS_1/DNS_2 im Walker-Carcinom der Ratte.

	Mengenverhältnis DNS_1/DNS_2
Unbestrahlter Tumor	1,7—3,5
Tumorgewebe, 2 Stunden nach Einwirkung von 5000 r Röntgenstrahlen entnommen	0,15—0,38

DISKUSSION

Aus den geschilderten Befunden geht hervor, dass die beiden nach dem Verfahren Bendichs aus dem ungestört wachsenden Walker-Carcinom isolierten DNS-Fraktionen gleich viel radioaktives Phosphat aufnehmen, sich stoffwechselmässig also nicht unterscheiden lassen. Nach Einwirkung von Röntgenstrahlen, die die Neubildung der DNS hemmend beeinflussen, zeigen DNS_1 und DNS_2 jedoch ein unterschiedliches Verhalten, indem die DNS_2 signifikant weniger Radiophosphor aufnimmt als die DNS_1. Der Effekt ist am ausgeprägtesten kurz nach der Bestrahlung und nach 12 Stunden noch gut nachweisbar, während nach 24 Stunden beide DNS-Fraktionen den

gleichen Gehalt an Radiophosphor aufweisen — wie der unbestrahlte Tumor. Diese Befunde bestätigen, dass die nach Bendich gewonnenen beiden DNS-Arten stoffwechselmässig keine einheitlichen Fraktionen darstellen; darüber hinaus zeigen sie, das der DNS-Umsatz im Walker-Carcinom durch die Röntgenstrahlen nicht gleichmässig getroffen wird.

Bei der Untersuchung verschiedener Organe der Ratte beobachteten Bendich und Mitarb., dass das Mengenverhältnis DNS_1/DNS_2 u. a. von der Zellteilungsrate abhängt. So liess sich aus der normalen Rattenleber keine DNS_1 gewinnen, wohl aber aus dem regenerierenden Organ nach vorangegangener partieller Hepatectomie [6]. Wie die eigenen Versuche ergaben, führen andererseits Röntgenstrahlen zu einer Minderung des DNS_1-Anteils im Gewebe (Tab. II). Es wird demnach offenbar das Mengenverhältnis DNS_1/DNS_2 durch Anregung der Zellteilung erhöht, während es umgekehrt durch Einwirkung ionisierender Strahlen, die die Mitose hemmen, beträchtlich kleiner werden kann. Der scheinbare Widerspruch, dass gleichzeitig die Hemmung der Radiophosphoraufnahme in der DNS_2 grösser ist als in der DNS_1, kann vorerst nicht erklärt werden.

Nach den ersten Untersuchungen durch von Euler und von Hevesy [9, 10] wurde der hemmende Effekt ionisierender Strahlen auf den Einbau von Radiophosphor in die DNS durch zahlreiche weitere Autoren beobachtet (u. a. [2, 14, 16, 20, 24, 28, 29, 30]). Bezüglich des Ausmasses der Hemmwirkung sind die Angaben unterschiedlich (Zusammenstellung bei von Hevesy [18, 19]). Gemeinsam ist jedoch allen Befunden, dass eine völlige Blockierung der DNS-Neubildung durch Röntgenstrahlen offenbar nicht möglich ist, auch nicht durch extrem hohe Strahlendosen, die mit Sicherheit weitere Zellteilungen im bestrahlten Gewebe verhindern. Eigene Untersuchungen am Mäusesarcom 180 zeigten, dass mit ansteigender Strahlendosis zunächst die DNS-Synthese im Tumor fortschreitend gehemmt wurde; oberhalb von etwa 1200 r führte jedoch eine Erhöhung der Dosis bis zu maximal 5000 r zu keinem weiteren Anwachsen der Hemmwirkung [13]. Gleichartige Beobachtungen machten Vermund, Barnum, Huseby und Stenstrom [30] bei Bestrahlungsversuchen an einem Mäuse-Mammacarcinom.

Die Tatsache, dass die DNS-Neubildung durch ionisierende Strahlen nur begrenzt gehemmt werden kann, legte bereits die Vermutung nahe, dass beim DNS-Umsatz möglicherweise verschiedenartige, im einzelnen noch unbekannte Stoffwechselvorgänge nebeneinander herlaufen und dass von diesen nur ein Teil strahlenempfindlich sei. Die nach Röntgenbestrahlung beobachtete unterschiedliche Aufnahme radioaktiven Phosphats durch DNS_1 und DNS_2 bildet eine weitere Stütze dieser Auffassung, nachdem zuvor die

Untersuchungen Bendichs gezeigt hatten, dass in der Tat die DNS eines Gewebes in ihrem umsatzmässigen Verhalten keine einheitliche Fraktion darstellt [4, 5, 6].

Zur Frage, an welcher Stelle innerhalb der Reaktionskette bei der DNS-Synthese die hemmende Strahlenwirkung einsetzt, geben Untersuchungen mit verschiedenartigen markierten Verbindungen erste Hinweise. So liess sich eine Hemmung der DNS-Neubildung mit Hilfe markierten Carbonats [27], ferner mit Acetat [17], Formiat [14, 15, 27], Glycin [1], Cytidin [22], 4-Amino-5-Imidazolcarboxamid [7] und Orotsäure [14] nachweisen. Bei Verabfolgung markierten Formiats beschränkte sich der Strahleneffekt jedoch auf eine Hemmung des Einbaues in die Purine und betraf nicht die Methylgruppe des Thymin [15]; auch die Aufnahme markierten Adenins durch die DNS blieb nach Röntgenstrahleneinwirkung unbeeinflusst oder wurde sogar gesteigert [14, 15, 23].

ZUSAMMENFASSUNG

Die von Bendich angegebene Methode zur Isolierung von zwei verschiedenartigen Fraktionen der Desoxyribonucleinsäure (DNS) wurde angewandt, um die Wirkung von Röntgenstrahlen auf die DNS-Synthese im Walker-Carcinom der Ratte mit Hilfe von Radiophosphor zu untersuchen. Im unbestrahlten Tumor wurde von beiden DNS-Fraktionen gleich viel radioaktives Phosphat aufgenommen. Nach Einwirkung von 5000 r Röntgenstrahlen war die Phosphataufnahme der DNS_2 zunächst stärker gehemmt als die der DNS_1. Andererseits wurde durch die Bestrahlung das Mengenverhältnis DNS_1/DNS_2 im Tumor beträchtlich verkleinert.

Der Deutschen Forschungsgemeinschaft, Bad Godesberg bei Bonn, danken wir für die Mittel, welche die Durchführung unserer Versuche ermöglichten.

SUMMARY

The effect of X-rays on DNA synthesis in the Walker carcinoma of the rat has been studied with the help of radiophosphorus, employing the method of Bendich for isolation of two different fractions of DNA. In the non-irradiated tumor, P^{32} was incorporated at equal rates into both DNA fractions. On irradiation with 5000 r of X-rays, the initial incorporation into DNA_2 was more inhibited than into DNA_1. On the other hand, by irradiation the ratio of the amounts of DNA_1/DNA_2 in the tumor was considerably decreased.

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THE APPLICATION OF INTERFEROMETRIC MICROSCOPY TO A QUANTITATIVE STUDY OF THE COLLOID IN THE THYROID GLAND OF THE GUINEA-PIG

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Histological observation in biology has been, until recent years, mainly of a qualitative character. Techniques such as ultraviolet microspectrography and autoradiography have been used quantitatively but the complicated, expensive and time-consuming nature of these has prohibited their general use in biological research and in routine pathological examination. In addition, their application is limited to the identification of a small number of substances.

Interferometric (interference) microscopy, on the other hand, has been shown to be an extremely valuable technique of wide application. It can be used to estimate the dry weight, refractive index, thickness and concentration of materials in isolated living and fixed cells [9, 2, 10, 3, 17, 18]. It has been used for the morphological examination of histological sections [1] and for the estimation of the dry weight and other values in them [6, 7]. Moreover, Davies, Engstrom and Lindstrom [8] have shown that the accuracy of the method compares favourably with the X-ray absorption technique of Engstrom and Lindstrom [13] and that it is simpler to use.

Previous work with interference microscopy was usually carried out for research purposes on fresh or frozen-dried material. It is not always possible or convenient to use frozen-dried or fresh material; thus it was felt that it would be of value to try to apply this technique to tissues prepared in a conventional histological manner.

Within limits the larger and more homogeneous the object the easier it is to examine by interference microscopy. The thyroid gland was an obvious choice as a test object. Brown, Ferguson and Ramsay [5] in this department have for some time been interested in the thyroid glands of guinea-pigs and I have made use of their material which had been prepared by routine histological methods to investigate the suitability of the technique. In addition, the investigation was designed to yield, if possible, quantitative information about the thyroid glands in relation to the animals from which they were obtained.

There were two groups of guinea-pigs. One group had been fed on a stock cube diet supplemented with greens and the other group on the same basal diet without greens but with a supplement of synthetic ascorbic acid. The point of the investigation was to see if there was any detectable difference in the mass per unit area of the colloid in the vesicles of the thyroids in the two groups.

The microscopic technique used here is essentially that developed by Davies, Wilkins, Chayen and LaCour [11]. It was, in the present instance, modified to permit the examination of tissue sections. The method depends on the fact that any object retards or accelerates light relative to the medium in which it is lying; the degree of this phase change is related to the amount of material, other than water, in the object and in the medium. This relation is a function of refractive index and of thickness. It is expressed as ϕ_w, the optical path difference in water measured in wavelengths (λ) of light. This path difference can be measured accurately in an interference microscope. When it is determined the amount of material in the object producing that change can be calculated from the basic formula

$$m = \frac{\phi_w A}{\chi}, \tag{1}$$

where m is the mass, A is the area and χ a constant related to the refractive index of the object.

Provided the limitations are realized the technique can be applied, with useful result, to sections prepared in conventional histological fashion.

MATERIALS

The care and treatment of the guinea-pigs from which the thyroids were obtained is described by Ferguson and Ramsay [15]. There were thirteen guinea-pigs, all females and approximately 260 days old. Eight had received a supplement of green vegetables, five a supplement of ascorbic acid. At death the mean weight of the guinea-pigs receiving the greens was 803 g, that of those receiving synthetic ascorbic acid was 627 g. The thyroid glands were removed from each animal after it had been killed by bleeding under ether anaesthesia. On removal, the glands of the guinea-pigs which had received the greens were larger.

The glands were fixed in 10 per cent neutral formalin for 24 h and then treated in the following fashion: 50 per cent methanol 8 h, 70 per cent methanol 18 h, 96 per cent ethanol (3 changes) 8 h, methyl benzoate and chloroform 18 h, methyl benzoate 2 h, benzene 2 h. The tissues were then embedded in paraffin wax for 1 h (3 changes). The blocks were numbered randomly by an independent observer in order to exclude any bias when quantitative measurements were being made. Sections were cut at 6μ on a flat cutting rotary microtome.

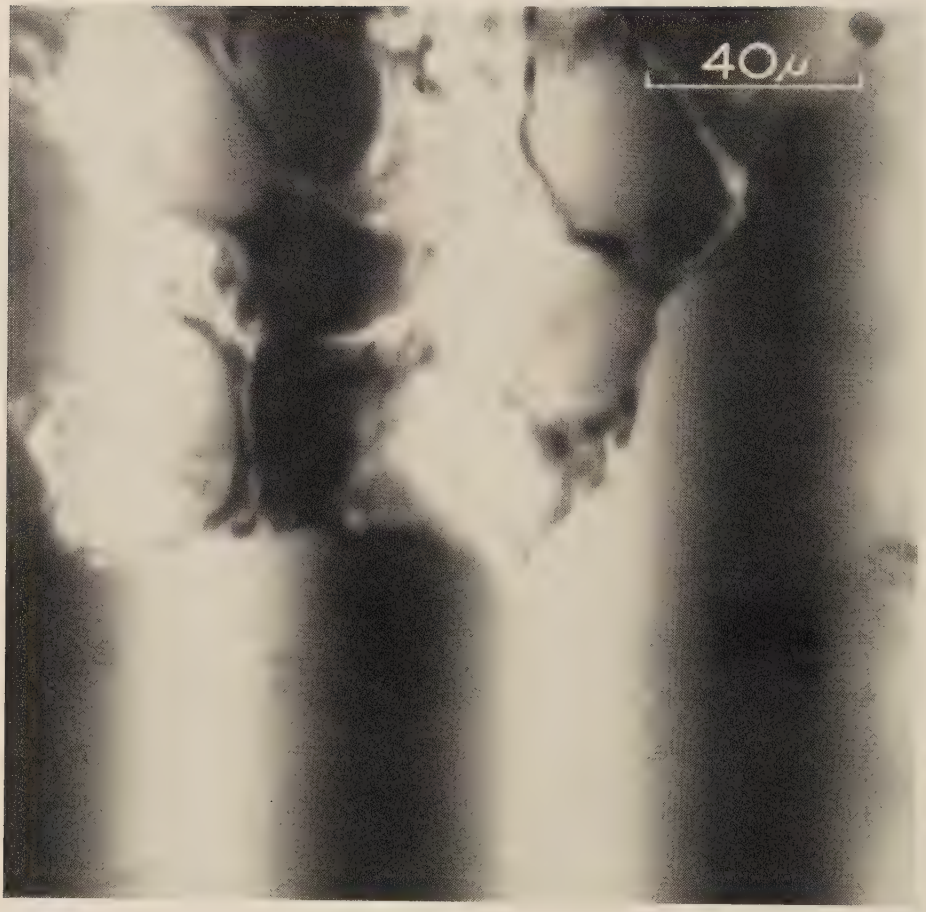
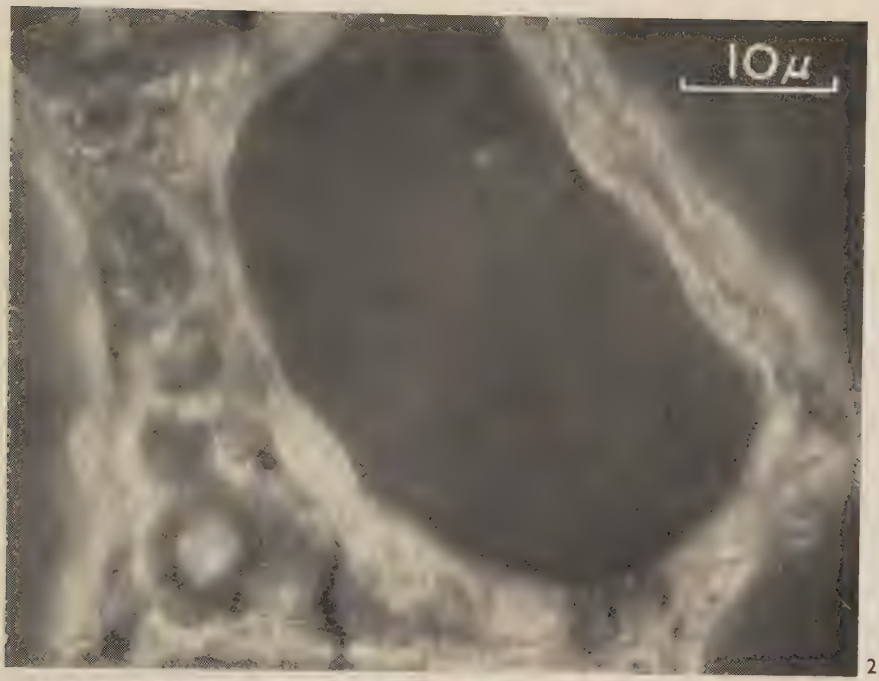


Fig. 1. A 6μ thick section of guinea-pig thyroid mounted in nonane and photographed with a small fringe separation. The lower part of the field is the background and it shows alternate black and white fringes. The distance from the centre of one black fringe to the centre of the next is one wavelength (=5461 Å). In the object, in the upper part of the field, the central black background fringe has been moved to the left. It has moved a distance of approximately one third of a wavelength. Similarly the other background fringes have been moved to the left. ×700.

Fig. 2 (top). A 6μ thick section of guinea-pig thyroid mounted in nonane and photographed at infinite fringe separation with the phase change of the colloid near minimum intensity. The lighter areas have a higher density. ×2000.

Fig. 3 (bottom). A 6μ thick section of guinea-pig thyroid mounted in nonane and photographed at infinite fringe separation with the phase change of the colloid near minimum intensity. The lighter areas have a higher density. The contrast is poor as the phtotograph was taken near the centre of a section where the comparison beam of the interference system is badly obstructed. The variation in optical retardation of the different vesicles is easily seen. ×875.



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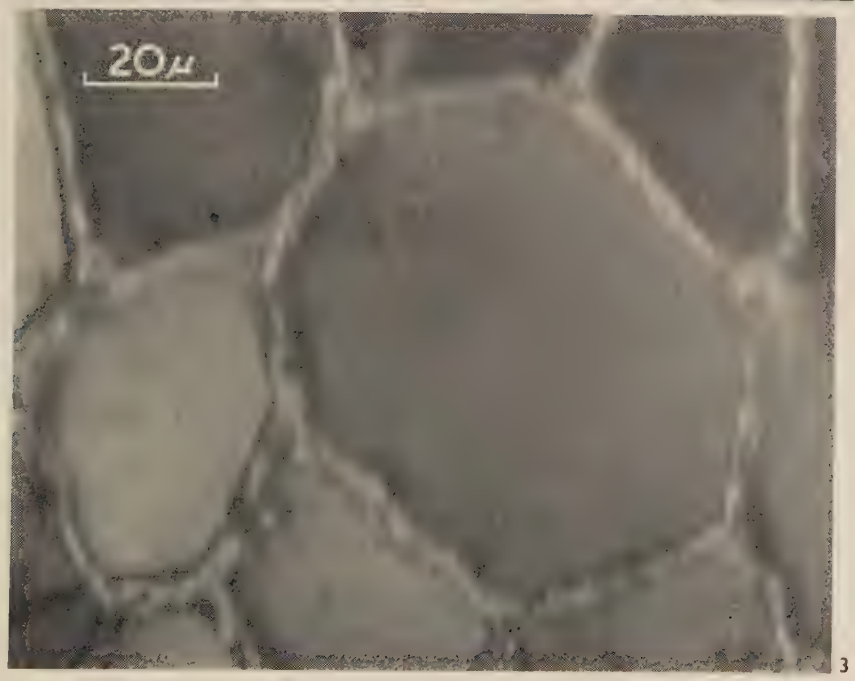


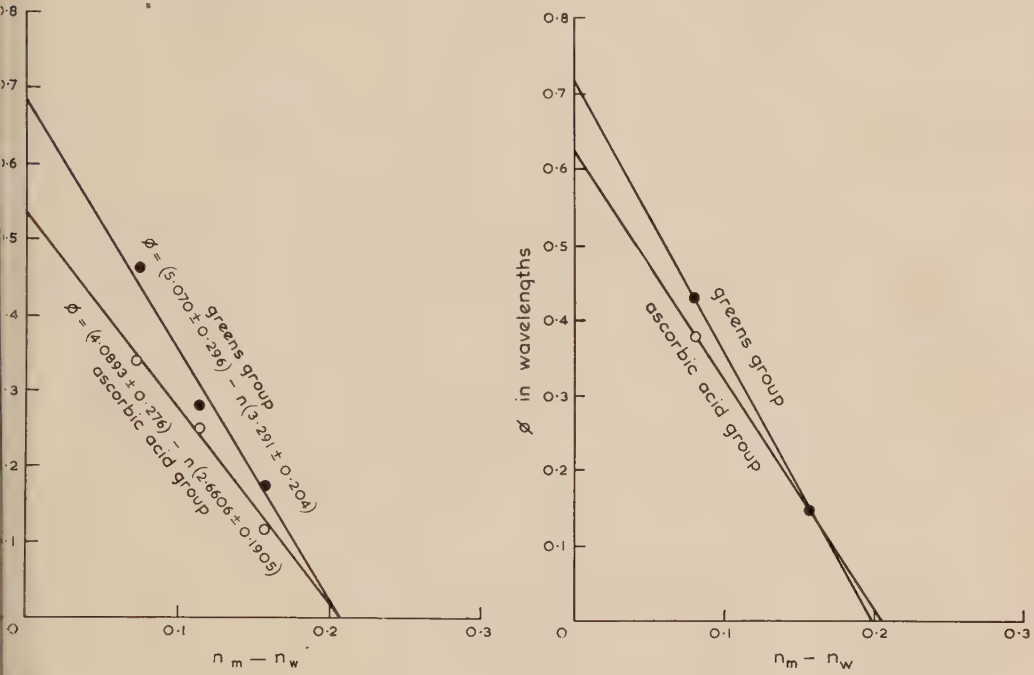
3

METHODS AND RESULTS

In the initial part of the investigation one lobe of the thyroid was examined from each animal. The paraffin sections were dry-mounted on glass slides at room temperature and treated immediately. Most of the original sections was cut away to leave only a small central area (1mm × 1mm). This prevented obstruction of the comparison beam in the interference system [11] and also minimised any variation in thickness which might occur at the edge of the section. Two sections, picked at random from a number cut from each block, were flooded, rinsed and mounted, one with nonane (refractive index 1.406) and the other with xylene (refractive index 1.490). These solutions remove the paraffin wax and provide suitable mounting media. Duplicate readings of the path differences produced by the colloid in 20 vesicles in each section were made, i.e. 320 observations for the greens group and 200 observations on the ascorbic acid group for each medium. The method was that described by Davies *et al.* [11] for the visual measurement of path difference. They transfer the condition of minimum intensity from the background, in an even field at infinite fringe separation, to the object under examination. The degree of accuracy obtainable with this method is around $1/50\lambda$. All measurements were made on a Cooke-Dyson microscope with an objective of 0.85 N.A. Illumination was according to Kohler, using green light of approximately 5461 Å isolated from a mercury vapour lamp by filters. When sections were examined in white light with interference fringes in the field it was seen that no vesicle produced a retardation as great as one wavelength in any of the media in which it was examined. The characteristic fringe shift produced by an area of colloid under these conditions is shown in Fig. 1. In the majority of vesicles the optical retardation was even throughout the colloid of that vesicle (Fig. 2). In some, however, there appeared to be an elliptical area of smaller retardation radiating outwards from one or two of the cells lining the vesicle. Chattering or scoring, when it occurred during sectioning, was easily detectable. Adjacent vesicles often showed marked differences in the optical retardation of the colloid present. This is shown in Fig. 3 where the lighter areas have greater retardation.

There was never any indication of an obvious variation in thickness across the sections examined. This would appear as a progressive alteration in optical retardation across the section irrespective of the varying optical retardations in the different vesicles in that section. There appeared to be no very definite relationship between the optical retardation of any area of

colloid and the size of the vesicle or the thickness of the lining epithelium which enclosed it. The curve, produced by plotting the mean values for ϕ for the two groups of animals against the refractive indices of the media, is shown in Fig. 4*a*. The significance of this will be discussed later.



Figs. 4*a* (left) and 4*b* (right). The lines in (*a*) are drawn through the mean values for ø obtained in nonane and in xylene in the first experiment. The lines in (*b*) are drawn through points calculated from the regression equations obtained in the second experiment. The points –○– and –●– represent the mean values obtained for the ascorbic acid and greens groups respectively, in nonane, chloroform and xylene. The regression equations for 4*b* are based on absolute refractive index.

The Effect of Histological Procedures on Tissues

It is certain that tissues are distorted by conventional histological methods but there is no sure way to evaluate the degree of this distortion. Observations on fixed tissues, using the technique of interference microscopy, cannot therefore be transferred directly to fresh tissues. Since, however, this technique gives quantitative results, it ought to be possible to measure with some degree of accuracy the actual degree and source of the distortions. The three main sources are probably 1. loss of material, from the colloid, during fixation and subsequent techniques; 2. variation in thickness of the sections; and 3. the effect of mounting media.

The mean mass per unit area of the colloid in the vesicles of the thyroid can then be determined by substituting the above values for t_1, together with the relevant value for ϕ, in equation (3). When ϕ is large so also is t_1; thus, the correction factor for thickness is more or less constant relative to ϕ. Since $n_m - n_w = 0$ when water is used as a mounting medium, no correc-

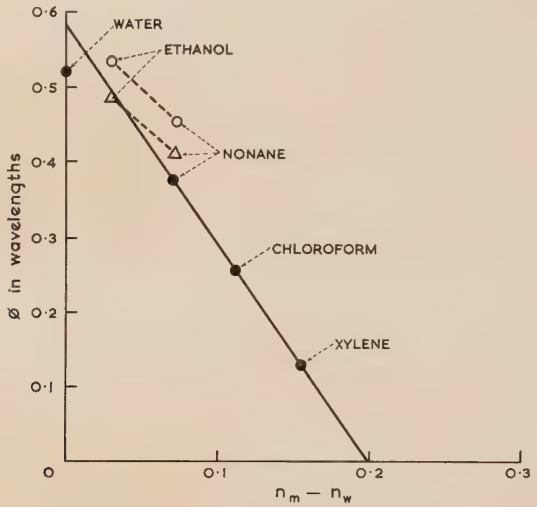


Fig. 5. Mean values – • – obtained for ϕ from animal No. 10 in the main experiment, when different sections cut at the same thickness were examined in water, in nonane, in chloroform, and in xylene. The symbols – ○ – and – △ – represent the values obtained for two vesicles examined first in nonane and then in ethanol after the nonane was washed off. The low values in ethanol, for these two vesicles, suggest that not all the nonane had been removed from the sections and it was thus producing a mounting medium with a refractive index midway between ethanol and nonane.

tion factor is required, and thus no determination of n_p or t_1 would be necessary if the sections were examined in water. However, if the sections are brought to water, the solubility of the colloid must be considered.

3. *The effect of mounting media on the colloid.*—Using the results obtained in non-aqueous media the value for ϕ, which would be given if the sections were mounted in water, can be obtained by extrapolation to the ordinate in Fig. 4*a*. If paraffin sections are treated with xylene, brought through alcohol to water, and then examined, the mean figure for ϕ is lower than would be expected. Fig. 5 shows the line drawn through the mean values for ϕ obtained from sections from the same block; these were mounted either in water, or in absolute alcohol, or in nonane, or in chloroform, or in xylene. The mean values in all media, except in water, lie on a straight line. The value for ϕ in water may have dropped because of loss of material from

the section during the hydrating process. Alternatively, the medium with a higher refractive index (xylene and ethanol) used in removal of the wax and in the hydration may not have been washed out by the water. Thus there may have remained round the colloid a medium with a refractive index higher than that of water.

To determine to what extent non-removal of the mounting media was influencing the result when sections were taken down to water, several sections were mounted in nonane under a coverslip resting on glass spacers. The optical retardations were measured; the coverslip was removed and the nonane washed off and replaced by absolute ethanol; the coverslip was replaced and the optical retardation measured on the same vesicles. The values for two representative vesicles are shown in Fig. 5. These values are jointed by interrupted lines. The slopes of the interrupted lines are much less steep than the slope of the continuous line joining the values obtained from a number of vesicles in different sections cut at the same thickness and examined in different media. The lower value obtained for representative vesicles in absolute ethanol is around the value which one would expect to obtain in a medium with a refractive index mid-way between that of nonane and that of ethanol. If the nonane is permitted to evaporate before the ethanol is added this drop in value does not occur. This suggests that there actually is a mixture of the two media round the colloid. Swelling of the colloid when mounted in water might cause a drop in the expected value for ϕ by redistribution of the material present. However, this does not seem to occur since planimetry of photographs, taken of the same vesicles in different media, showed that no swelling occurred in water.

After these various checks had been completed the initial series of observations was repeated. The sections were treated in an identical way and examined in media which removed the paraffin and also provided a medium of the desired refractive index. On this occasion sections from the other lobes of the original thyroid glands were examined in three different media instead of in two as in the original series. A random selection of five animals in the group receiving a supplement of greens was made in order to simplify the subsequent statistical analysis. Duplicate measurements were made of the optical retardations produced by five vesicles in each of the sections mounted in xylene, in nonane and in chloroform (refractive index 1.446). The result of their statistical analysis is given in Table II. The regression of the group receiving greens is

$$\phi = (5.070 \pm 0.296) - n\,(3.291 \pm 0.204)$$

The mean mass per unit area of the colloid in the vesicles of the thyroid can then be determined by substituting the above values for t_1, together with the relevant value for ϕ, in equation (3). When ϕ is large so also is t_1; thus, the correction factor for thickness is more or less constant relative to ϕ. Since $n_m - n_w = 0$ when water is used as a mounting medium, no correc-

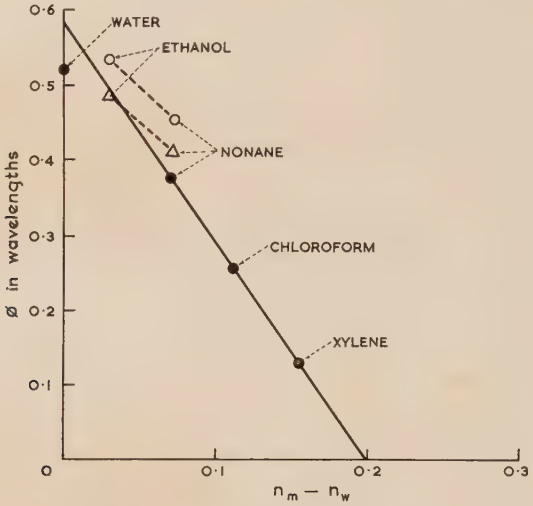


Fig. 5. Mean values – • – obtained for ϕ from animal No. 10 in the main experiment, when different sections cut at the same thickness were examined in water, in nonane, in chloroform, and in xylene. The symbols – ○ – and – △ – represent the values obtained for two vesicles examined first in nonane and then in ethanol after the nonane was washed off. The low values in ethanol, for these two vesicles, suggest that not all the nonane had been removed from the sections and it was thus producing a mounting medium with a refractive index midway between ethanol and nonane.

tion factor is required, and thus no determination of n_p or t_1 would be necessary if the sections were examined in water. However, if the sections are brought to water, the solubility of the colloid must be considered.

3. *The effect of mounting media on the colloid.*—Using the results obtained in non-aqueous media the value for ϕ, which would be given if the sections were mounted in water, can be obtained by extrapolation to the ordinate in Fig. 4*a*. If paraffin sections are treated with xylene, brought through alcohol to water, and then examined, the mean figure for ϕ is lower than would be expected. Fig. 5 shows the line drawn through the mean values for ϕ obtained from sections from the same block; these were mounted either in water, or in absolute alcohol, or in nonane, or in chloroform, or in xylene. The mean values in all media, except in water, lie on a straight line. The value for ϕ in water may have dropped because of loss of material from

the section during the hydrating process. Alternatively, the medium with a higher refractive index (xylene and ethanol) used in removal of the wax and in the hydration may not have been washed out by the water. Thus there may have remained round the colloid a medium with a refractive index higher than that of water.

To determine to what extent non-removal of the mounting media was influencing the result when sections were taken down to water, several sections were mounted in nonane under a coverslip resting on glass spacers. The optical retardations were measured; the coverslip was removed and the nonane washed off and replaced by absolute ethanol; the coverslip was replaced and the optical retardation measured on the same vesicles. The values for two representative vesicles are shown in Fig. 5. These values are jointed by interrupted lines. The slopes of the interrupted lines are much less steep than the slope of the continuous line joining the values obtained from a number of vesicles in different sections cut at the same thickness and examined in different media. The lower value obtained for representative vesicles in absolute ethanol is around the value which one would expect to obtain in a medium with a refractive index mid-way between that of nonane and that of ethanol. If the nonane is permitted to evaporate before the ethanol is added this drop in value does not occur. This suggests that there actually is a mixture of the two media round the colloid. Swelling of the colloid when mounted in water might cause a drop in the expected value for ϕ by redistribution of the material present. However, this does not seem to occur since planimetry of photographs, taken of the same vesicles in different media, showed that no swelling occurred in water.

After these various checks had been completed the initial series of observations was repeated. The sections were treated in an identical way and examined in media which removed the paraffin and also provided a medium of the desired refractive index. On this occasion sections from the other lobes of the original thyroid glands were examined in three different media instead of in two as in the original series. A random selection of five animals in the group receiving a supplement of greens was made in order to simplify the subsequent statistical analysis. Duplicate measurements were made of the optical retardations produced by five vesicles in each of the sections mounted in xylene, in nonane and in chloroform (refractive index 1.446). The result of their statistical analysis is given in Table II. The regression of the group receiving greens is

$$\phi = (5.070 \pm 0.296) - n\,(3.291 \pm 0.204)$$

TABLE II

Analysis of variance of 300 observations of ϕ in the second experiment.

Source of variance	Sum of squares	Degree of freedom	Mean square	Variance ratio (F)	Probability (P)
Joint regression	3.1256	1			
Difference between regressions	0.0352	1	0.035	7	<0.01
Sum of separate regressions	3.1608	2			
Deviations from linearity					
Greens	0.0666	1	0.067	13	<0.001
Ascorbic acid	0.0057	1	0.006	1.2	n.s.
Between animals					
Greens	0.4274	4	0.107	21	<0.001
Ascorbic acid	0.0717	4	0.018	3.6	<0.05
Jointly	0.4991	8	0.062	12.4	<0.001
Residual (within animals corrected for regression, linearity and between animals)	1.4649	286	0.005		
Total about means	5.1971	298			
Difference between means	0.3509	1	0.351	70.2	<0.001
Total	5.5480	299			

and that for the group receiving ascorbic acid is

$$\phi = (4.0893 \pm 0.276) - n\,(2.6606 \pm 0.1905).$$

The regression lines drawn from these equations (Fig. 4*b*) are significantly different with respect to both coefficients (slopes) and means and they correspond closely to those drawn through the mean values for ϕ obtained in the two media in the initial experiment (Fig. 4*a*). The refractive index of the colloid, as obtained by extrapolation to the base line, is again 1.54.

DISCUSSION

Figs. 4*a* and 4*b* show that there is an easily detectable difference in the values for optical retardation of the colloid in the two groups of animals. Conversion of these values to mass gives a mean value of mass per unit area of 2.2 $\mu\mu g/\mu^2$ for the group receiving greens and of 1.6 $\mu\mu g/\mu^2$ for the ascorbic acid group [14]. The range of values is 0.8–3.4 $\mu\mu g/\mu^2$ and 0.7–

3.1 $\mu\mu g/\mu^2$ respectively. The nutritional significance of this difference will be discussed in relation to the diet in a separate publication [15].

Davies, Engstrom and Lindstrom [8] give values for the mass of the colloid in four vesicles of the thyroids of rats which range from 1.4–2.0 $\mu\mu g/\mu^2$. The tissue used by them was frozen-dried and cut at 6μ (personal communication). In the present series tissue fixed in formalin and embedded in paraffin gave similar values when cut at 6μ. Frozen-dried tissue cut at 6μ gave a mean value 35 per cent higher than the average obtained from the entire formalin-fixed series, and from the formalin-fixed half of the same thyroid gland. The values reported by Davies, Engstrom and Lindstrom fall within the wide range obtained from both the formalin-fixed and the frozen-dried series and thus it is not safe to generalise about the significance of a comparison. In addition there is a possible species difference.

When frozen-dried sections were extracted with buffer at pH 7 approximately 75 per cent of the colloid was removed. According to Gersh [16] this extractable material is thyroglobulin and the material left is a glycoprotein. The 35 per cent loss of material during formalin fixation may all be thyroglobulin or may not and further work is being done to investigate this. This degree of loss during formalin fixation corresponds to that reported by Engstrom and Glick [12] and by Sylvén [20] for different tissues.

In the thyroid used for the experiment on the influence of fixation the values for colloid in the part fixed in formalin correspond to those obtained in the main series. It thus appears that, although there is a loss of material during formalin fixation, this loss is relatively constant.

The refractive index of the colloid in both experiments and in both series of animals, was 1.54. This value is identical with that obtained previously for the particles in fixed sperm heads [11]. The concentration of material other than water in the particles of colloid can be calculated from the formula

$$\frac{n_p - n_w}{\chi} = c, \tag{6}$$

where c is the concentration in g per unit volume. Thus the concentration of material present in the particles is 114 g/100 ml.

In a study such as this a comparison of the path differences produced by the colloid in different sections relies on the microtome cutting at a constant thickness: it is not necessary to know what this thickness is in order to calculate the mass per unit area. If the path differences are measured using water as a mounting medium then no determination of geometric thickness (t) is required. If the path differences in an object permeated by the mounting

medium are measured in any medium other than water then the effective (optical) thickness (t_1) of the object must be calculated before the mass per unit area can be determined (eqn. 3). If the effective thickness is expressed as a fraction of the geometric thickness then the volume of the layer of colloid occupied by the material in that layer can be determined.

$$2\mu/6\mu = \tfrac{1}{3} = 33 \text{ per cent } (1 - f \text{ in eqn. (3)}).$$

This volume is, however, dependent on knowing the geometric thickness, which cannot be determined easily. Assuming that these sections were cut at a constant thickness of 6μ then the average fraction of the volume actually occupied by material is 31.7 per cent (the range is 10–50 per cent) in the group receiving greens. In the ascorbic acid group the corresponding value is 23.3 per cent and the range is 9–45 per cent. As the concentration of material in the particles themselves has already been shown to be 114 g/100 ml then the concentration of material in the colloid can be calculated. Thus, in the group receiving greens the average concentration is 35 g/100 ml and in the ascorbic acid group it is 26.6 g/100 ml. It is hoped that no systematic variation in geometric thickness has been introduced into the two series and the methods of reducing this possible variation have been described. Even if the geometric thickness is constant throughout the two series it is not possible to say what it is and thus any determinations of the concentration of material present, as described above, are open to doubt.

It is possible that error may be introduced in carrying sections through alcohol to water; thus it is safer to examine the sections in a medium which removes the paraffin and also provides the required refractive index for observation.

By interferometric microscopy it is possible to compare different histological sections prepared in a conventional fashion and to obtain information about the amount of material in them. However, care must be taken in planning the investigation to eliminate or minimise the sources of error.

A more detailed investigation might reveal a relationship between mass per unit area and the height or volume of the epthelial lining or the true areas of the vesicles in which the measurements are made. Thus a simple method of estimating thyroid activity might be obtained.

SUMMARY

1. Two groups of guinea-pigs were maintained on the same basal diet without vitamin C; one group received a supplement of fresh green vegetables daily while the other group received synthetic ascorbic acid daily.

2. The thyroids which were removed from these animals were prepared by conventional histological methods and examined by interferometric microscopy.

3. The mass of material present in the colloid in vesicles of these glands was determined. The mean value of mass per unit area of the colloid in the thyroids of the group receiving greens was 2.2 $\mu\mu g/\mu^2$. In the ascorbic acid group this value was 1.6 $\mu\mu g/\mu^2$. The difference in values is significant statistically.

4. When sections prepared in conventional fashion are used for interferometric microscopy there are possible sources of error associated with variation in the thickness of the section, with losses during fixation and with the effect of solvents. These sources of error have been systematically investigated and the findings are reported.

To Professor R. C. Garry and Dr. H. S. D. Garven for their interest and encouragement and to Dr. H. G. Davies for his constructive criticism I would like to express my thanks. I would also like to thank Dr. S. D. Morrison for his help in planning and carrying out the statistical analysis, and Dr. J. B. de V. Weir for checking the result.

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STUDIES ON THE FUNCTION OF INTRACELLULAR RIBONUCLEASES

I. THE ACTION OF COBALT AND NICKEL ON *TETRAHYMENA PYRIFORMIS* W[1]

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Cobalt in various forms has many diverse effects in the animal organism and on animal and plant cells. Levy, Skutch and Schade [9] found that *Proteus vulgaris* placed in the presence of Co^{++} continued to synthesize ribonucleic acid (RNA) but not deoxyribonucleic acid (DNA) or protein. Miura and Nakamura [11] confirmed this action of Co^{++} using *Staphylococcus aureus* and *Escherichia coli*, and from studies on the action of Co^{++} on crystalline pancreatic ribonuclease (RNase) concluded that the inhibition of intracellular RNase accounted for the observed effects. Recently Heath [6] has reported that certain concentrations of Co^{++}, when added to actively growing chick heart fibroblasts *in vitro* cause abnormal mitoses in which the nucleoli persist throughout the whole cycle, and finally, with the formation of the new nuclei in telophase, are left in the cytoplasm of the daughter cells.

It would seem reasonable to explain the above effects of Co^{++} as being due to the inhibition of intracellular RNases by this ion. Inhibition of this enzyme (or enzymes) could conceivably result in the accumulation of RNA in the bacterial cells studied, or in the failure of the RNA containing nucleoli to be broken down during mitosis. As it has been reported [13] that another, quite different, RNase inhibitor heparin [14, 19] may also often cause the accumulation of RNA in cells in tissue culture, this would appear to be further support for the explanation first suggested by Miura and Nakamura. However, there are certain difficulties with this explanation which seemed to us to warrant further study. We have not been able to confirm Miura's and Nakamura's results [13] with regard to the action of Co^{++} and Ni^{++} on crystal-

[1] A portion of this material was presented as an abstract at the Seventh Annual Meeting of the Society of Protozoologists, East Lansing, Michigan, September 1955.

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line pancreatic RNase and furthermore, since this enzyme is apparently quite different from intracellular RNases [8, 12, 15], conclusions based on the effect of Co^{++} on the crystalline enzyme would not be valid. We have, therefore, investigated in some detail the effects of Co^{++} on the protozoan *Tetrahymena pyriformis* W. For comparison Ni^{++}, which is quite similar to Co^{++} in chemical properties but does not exhibit the same biological action at comparable concentrations, has been studied similarly. Experiments with crystalline pancreatic RNase have also been carried out.

MATERIALS AND METHODS

Tetrahymena pyriformis W was cultured in 2 per cent peptone containing 0.2 per cent Marmite (yeast preparation). For growth studies cells were grown in slanted test tubes containing 5.0 ml of medium. Optical densities were determined at appropriate intervals in a photoelectric colorimeter. Cobalt and nickel were determined by the method of Chilton [2]. When organic material was present, samples were ashed in air and the residue taken up in hot concentrated HCl. The Schneider procedure [17] was utilized to prepare a mixture of RNA and DNA nucleotides from cells, omitting extraction of lipids from the trichloroacetic acid (TCA) precipitate. DNA was measured by the method of Dische [3]. RNA was determined by difference after determination of the total absorption of the hot TCA extract at 260 mμ. RNase was determined by a modification, previously described [14], of the method of McCarty [10]. Nitrogen was estimated by a micro-Kjeldahl procedure. Crystalline pancreatic RNase was obtained from Worthington Biochemical Sales Co., Freehold, N.J.

RESULTS

In Vivo Effects of Co^{++} and Ni^{++}

The effect of Co^{++} and Ni^{++} on the growth of Tetrahymena.—Cobalt and nickel as the sulfates were added to media in test tubes which were then autoclaved at 15 lbs for 20 minutes. Three test tubes for each concentration were prepared and one tube, which was uninoculated, served as a control. One loopful of 3 day W strain was transferred into the tubes which were then kept in the dark at room temperature in a slanted position. Tubes containing more than 1×10^{-4} *M* Co^{++} turned darker with time, possibly due to reaction of Co^{++} with histidine and oxygen in the medium [5]. The amount of Co^{++} and Ni^{++} originally present in the medium was less than 0.1γ per ml while there was 0.2γ of Cu^{++}. The results of the growth experiments are shown in Figs. 1 and 2. Co^{++} at all levels used inhibited the initial growth rate, but with concentrations ranging from 5.9 to 17.7γ per ml the final growth was greater than the controls. A concentration of 23.6γ per ml greatly

increased the lag period and reduced the final growth at 216 hours to one-third that of the controls. There was no growth in tubes containing higher concentrations of Co^{++}. These higher concentrations (up to 47.2γ per ml) were not lethal to the cells, for the original inoculum remained viable for at least 240 hours. In another experiment Co^{++} was added to a culture of

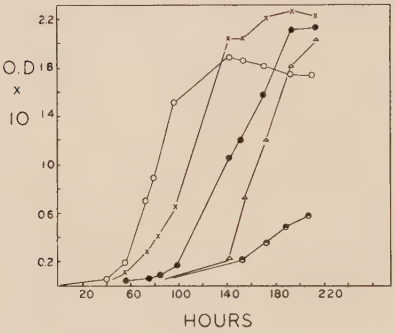

Fig. 1. The effect of Co^{++} on the growth of *Tetrahymena pyriformis* W in peptone medium.

○——○ Controls
×——× 5.9 γ Co^{++} per ml
●——● 11.8 γ „ „ „
△——△ 17.7 γ „ „ „
◓——◓ 23.6 γ „ „ „

rapidly growing *Tetrahymena* to give a concentration of 84γ per ml. The culture of approximately 10^5 cells per ml contained many cells in division. Three hours after adding the Co^{++}, as far as could be determined by microscopic inspection all of the cells remained viable but there were no further divisions.

Slater [18] has determined that *Tetrahymena pyriformis* E has a nutritional requirement for Co^{++}. He examined the effect of addition of Co^{++}, up to a maximum of 8γ per ml, to a Co^{++}-free synthetic medium and reported that as little as 0.1γ Co^{++} per ml gave good growth, while higher concentrations (4 and 8γ per ml), in agreement with the present results, slowed the initial growth rate, but gave a final higher population density.

The effect of Ni^{++} on the growth of *Tetrahymena* is depicted in Fig. 2. Concentrations up to 17.7γ per ml had little significant effect on the growth rate. In the range from 17.7 to 41.3γ per ml there was an increasing lag in the initial growth but after about 120 hours the growth in the tubes containing this range of concentrations somewhat exceeded that of the controls. A Ni^{++} concentration of 47.2γ per ml was inhibitory, the final population density was less than one half that of the controls at 120 hours while 59γ per ml allowed only slight growth in the same period.

Co^{++} was considerably more inhibitory, therefore, to the growth of *Tetrahymena* than equivalent concentrations of Ni^{++}. The effect of the metals appears to be mainly an inhibition of cell division as the highest concentrations employed were not lethal to the cells. The differences observed may be

due to variations in permeability of the cell wall to these two ions. Since Co^{++} apparently is required by the protozoa, while Ni^{++} is probably not essential, the former ion may pass more readily through the cell membrane giving rise to higher concentrations of Co^{++} within the cell. Ni^{++} may be largely excluded even when present in high concentrations in the medium.

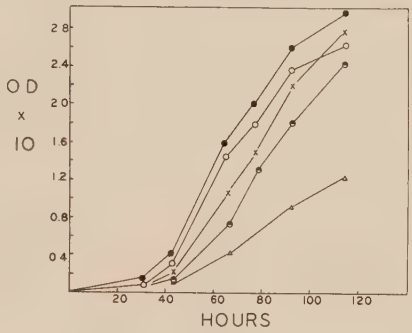

Fig. 2. The effect of Ni^{++} on the growth of *Tetrahymena pyriformis* W in peptone medium.

○———○ Controls
●———● 17.7 γ Ni^{++} per ml
×———× 29.5 γ „ „ „
◓———◓ 41.3 γ „ „ „
△———△ 47.2 γ „ „ „

The effect of Co^{++} and Ni^{++} on the RNA and DNA content of Tetrahymena.—Three 4-liter amber bottles, containing 750 ml of peptone-Marmite medium, were inoculated with 1.0 ml of 5- or 7-day-old W strain and placed on their sides. The cells were allowed to multiply at room temperature for 3 days after which Co^{++} and Ni^{++} sulfates were added, respectively, to two of the bottles to give a concentration of 29.5γ of metal ion. All three bottles were then incubated for an additional 24 hours in two experiments and 48 hours in the third experiment. At the end of the incubation period, the cells in all three bottles were counted, collected by gentle centrifugation, washed three times with distilled water and made up to a volume of 25.0 ml. The protozoa treated with Co^{++} were distinctly browner than the controls or Ni^{++} treated cells. Five ml aliquots of the concentrated cell suspension were immediately treated by the Schneider procedure and aliquots were also taken for nitrogen determinations, dry weights and metal content. The results are given in Table I. In experiments 1 and 2 in which the protozoa were in contact with the metal salts for 24 hours, there were 17.8 and 26.1 per cent fewer cells in the Co^{++} containing media, respectively, while in experiment 1, Ni^{++} depressed the cell count by only 11.3 per cent and in experiment 2 there were 3 per cent more cells in the Ni^{++} containing medium. In keeping with this, dividing cells were noted in the controls and Ni^{++} containing media, but not in those containing Co^{++}. In spite of the decrease in cell count in the Co^{++} containing media, the amounts of N, RNA, DNA, and the dry weights of these cells were not greatly different from the controls. For the

TABLE I

The effect of Co^{++} and Ni^{++} on the nucleic acid and nitrogen content of *Tetrahymena*.

Experiment No.	Cells/ml $\times 10^{-3}$	Dry wt/ml† mg	N content/ml mg	RNA/ml mg	DNA/ml μg	Metal ion/ml μg	RNA/cell $\mu g \times 10^2$	DNA/cell $\mu g \times 10^3$
I. 24 hrs in presence of 29.5 γ/ml metal ion								
1. Control	169	17.0	1.64	2.27	348		1.34	2.06
2. Co^{++} treated	139	16.9	1.58	2.22	356	5.55	1.60	2.57
3. Ni^{++} treated	150	17.8	1.64	2.30	340	3.81	1.53	2.26
II. 24 hrs in presence of 29.5 γ/ml metal ion								
1. Control	194	17.6	1.68	2.55	376		1.31	1.94
2. Co^{++} treated	120	16.6	1.66	2.31	402		1.92	3.35
3. Ni^{++} treated	200	19.4	1.71	2.75	451		1.38	2.25
III. 48 hrs in presence of 29.5 γ/ml metal ion*								
1. Control	195	21.7	2.16	2.89	430		1.48	2.20
2. Co^{++} treated	103	10.1	0.94	1.31	280		1.27	2.62
3. Ni^{++} treated	174	19.8	1.96	2.79	328		1.60	1.89

* 7-day culture used for inoculation. In exp. I and II a 5-day culture was used.
† Dried at 80° for 24 hrs.

Ni^{++} treated cells the corresponding values were the same or slightly higher than the controls. As a result, the values of RNA and DNA per cell were considerably higher for the Co^{++} treated and somewhat higher for the Ni^{++} treated, than the controls. Thus Co^{++}, and to a lesser extent Ni^{++}, appear to interfere with the normal turnover, in this case presumably the breakdown by nucleases, of nucleic acids. The effect of the ions on the nuclease activity of cell free homogenates is reported in Section II of this paper.

In experiment 3, DNA per cell was also elevated but RNA per cell was less than in the controls, suggesting that other factors are beginning to operate in cells exposed to Co^{++} for 48 hours. Possibly the cells are deteriorating after this length of exposure. It is of interest to note, however, that the ratio of RNA to N is remarkably constant in all experiments in contrast to the results with bacteria [9], and it appears that protein synthesis is not greatly inhibited in *Tetrahymena* by the concentration of Co^{++} used in this experiment. In experiment 1, assay of the concentrated cell suspensions for cobalt and nickel indicated that relatively large amounts of these metals were inside or adsorbed on the surface of the cells. Present techniques do not allow us to distinguish

TABLE II

The effect of various concentrations of Co^{++} and Ni^{++} on the activity of crystalline pancreatic ribonuclease.

	Average of triplicate experiments			
	Final conc. of metal ion	Amount of enzyme per 25 ml soln.	Inhibition by Co^{++}	Inhibition by Ni^{++}
	M	γ	per cent	per cent
1	1.6×10^{-4}	2	6	9
2	4×10^{-4}	2	17	28
3	8×10^{-4}	2	31	56
4	1.6×10^{-3}	2	52	72
5	8×10^{-4}	1	52	64

the extent of these two possibilities. Approximately 30 per cent more cobalt than nickel was taken up (somewhat greater than this on a per cell basis) by the protozoa.

In Vitro Effects of Co^{++} and Ni^{++}

The action of Co^{++} and Ni^{++} on crystalline pancreatic RNase.—The effect of various concentrations of Co^{++} and Ni^{++} sulfates on pancreatic RNase is given in Table II. The experimental conditions were as follows: Ten ml of 0.2 per cent NaRNA were incubated with 1.0 ml of metal salt solution for 10 minutes and then 10 ml of 0.1*M* veronal-acetate buffer, pH 7.8, and 4.0 ml of enzyme solution were added. Aliquots were removed at two-minute intervals and precipitated with an equal volume of 1*N* HCl. The turbidity was read after three minutes in a photoelectric colorimeter. Controls utilizing 1 ml of H_2O instead of salt solution were run similarly. The salts had no effect on the precipitability of the RNA by HCl and incubation of them with the substrate for longer periods of time did not alter the results. The per cent inhibition was calculated from the ten-minute reading.

Examination of the data shows that Ni^{++} was more inhibitory than Co^{++} at all concentrations used, amounting to 72 per cent with 1.6×10^{-3} *M* $NiSO_4$. Decrease in enzyme concentration increased the per cent inhibition by Co^{++} markedly and by Ni^{++} slightly. Miura and Nakamura [11] reported that 3×10^{-3} *M* $CoCl_2$ inhibited crystalline pancreatic RNase by 12 per cent, while $NiSO_4$ at the same concentration had no effect. They used the Klein method of assay [7] but did not report the details of their experimental conditions

such as substrate and enzyme concentration. It is possible that the differences between their work and the present report may be due to large differences in experimental conditions, particularly in enzyme concentration, as this appears to be an important factor. Not enough experimental data are presented in Table II to determine whether the inhibition is due to a combina-

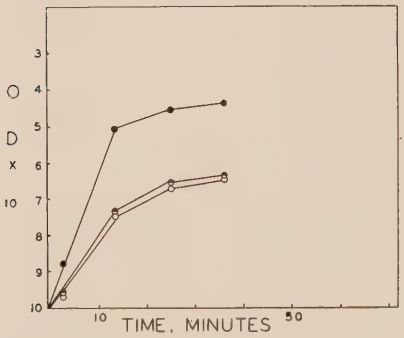

Fig. 3. The effect of Co^{++} and Ni^{++} on the activity of RNase in homogenates of *Tetrahymena pyriformis* W.

● ——— ● Control
◓ ——— ◓ 8.0×10^{-4} *M* Ni^{++}
○ ——— ○ 8.0×10^{-4} *M* Co^{++}

tion of Co^{++} and Ni^{++} with the enzyme, substrate or both. Since the inhibition of pancreatic RNase by Mg^{++} has been attributed to the formation of a substrate-metal complex, the inhibition by Co^{++} and Ni^{++} is probably of this nature, although combination with the enzyme cannot be ruled out. Recent studies on the structure of pancreatic RNase indicate that there are no free SH groups [1] with which the metal ions might combine. As previously mentioned, the fact that Co^{++} or Ni^{++} inhibit pancreatic RNase has no bearing on the possible effects of these metal ions on other intracellular RNases which may be quite different in their properties from the pancreatic enzyme [8, 12, 15]. Studies were carried out, therefore, on the effect of Co^{++} and Ni^{++} on the RNase activity in cell-free homogenates of *Tetrahymena*.

The effect of Co^{++} and Ni^{++} on RNase activity in Tetrahymena homogenates.—Three-day cultures of *Tetrahymena pyriformis* W were grown and harvested as described above. The 25 ml concentrates were homogenized to cell-free preparations by the method described by Eichel [4] and Co^{++} and Ni^{++} added to aliquots of the homogenates to give a final concentration of 8.0×10^{-4} *M*. Assays for RNase were performed by the modified McCarty procedure [10] at pH 6.1 using veronal-acetate buffer. The results of a typical assay are plotted in Fig. 3. It may be seen that both Co^{++} and Ni^{++} were inhibitory to RNase activity to about the same degree at this concentration in distinction from the effects of the ions on pancreatic RNase where Ni^{++} was considerably more inhibitory.

DISCUSSION

When testing the effects of various substances such as metal ions on enzyme activity in crude homogenates from cells, it is difficult to assess the effects of these substances on the intact cell where the relation of the components is undisturbed, membranes are intact and active absorption or exclusion of physiologically active substances may be in progress. In addition, in some cells, the presence of two RNases and an inhibitor for at least one of them has been detected [8, 15, 16]. However, in *Tetrahymena*, no studies have been made concerning the presence of more than one RNase or an inhibitor. It is true also, that investigations of the effect of metal ions or other substances on purified enzymes and enzyme systems may be criticized on the basis that, in the cell, the components are in the presence of a complex and carefully organized milieu in which there may be many interactions. In spite of these difficulties the data presented are, at least, not inconsistent with the theory that in *Tetrahymena* the inhibitory effects of Co^{++} and Ni^{++} on cell division are due to the inhibition of intracellular RNase activity, and perhaps DNase activity, which in turn prevents the normal breakdown and turnover of nucleic acids. The fact that more Co^{++} than Ni^{++} is taken up by the cells could explain the greater activity of the former ion.

SUMMARY

The effect of Co^{++} and Ni^{++} on the growth, RNA and DNA content of *Tetrahymena pyriformis* W was determined. Co^{++} was considerably more inhibitory to growth than equivalent concentrations of Ni^{++} and the former ion resulted in a significantly increased RNA and DNA content per cell under certain conditions. The action of Co^{++} and Ni^{++} on crystalline pancreatic RNase and on RNase activity in *Tetrahymena* homogenates was also determined and the results discussed in terms of a possible function of intracellular RNase.

The author would like to express his sincere appreciation to Dr. M. Webb for his encouragement and aid in this study and to Dr. Honor B. Fell for her interest and provision of facilities for doing this work.

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A RECORDING MICROSPECTROPHOTOMETER

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Since Caspersson's pioneer work in the field of microspectroscopy, several complex electrical and optical instruments have been devised in order to study the absorption of intracellular components, notably by Caspersson himself [1], by Wyckoff [6] at the Massachusetts Institute of Technology, by Keohane [2] at Bristol University, and by Pollister and Ornstein [3] in New York. All these are of the photoelectric type, and in addition a photographic recording instrument has been made at King's College London [4] which is used together with a suitable microdensitometer to make many measurements on living and fixed cells. While this photographic instrument is convenient for recording the total amounts of absorbing substances, it was felt that if the method were to be extended to a wider range of cellular components an instrument would be required which would conveniently record complete absorption spectra over a wide spectral range. We might then hope to investigate small shifts in spectra and to identify more precisely the substances present.

The microspectrophotometer, which is now complete, has, like the other recording instruments mentioned, an optical system with two light paths, one of which passes through the specimen. The intensity of the specimen beam is then compared with that of the second reference beam. This arrangement compensates for variations in the emissivity of the source throughout the spectrum, and when, as in the present instance, the two beams are recombined on to the cathode of a single photomultiplier, errors due to varying photocathode sensitivities are eliminated. In contrast, however, to the electrical methods of comparing intensities favoured by other authors, we have used an optical attenuator to balance the energy in the two beams which are made to fall alternately on to the photomultiplier. This attenuator is continuously adjusted into the balance position by a servo-mechanism whose input is the A.C. signal which is present at the anode of the photomultiplier when the two beams have different intensities. Thus the accuracy of the

method will depend on optical and mechanical design and will be virtually independent of electrical characteristics.

We have also been able to use a simple but efficient monochromator, since only a small aperture (f/80) is required to illuminate the solid quartz reflecting objective of Wilkins and Norris [5] that is employed as a condenser.

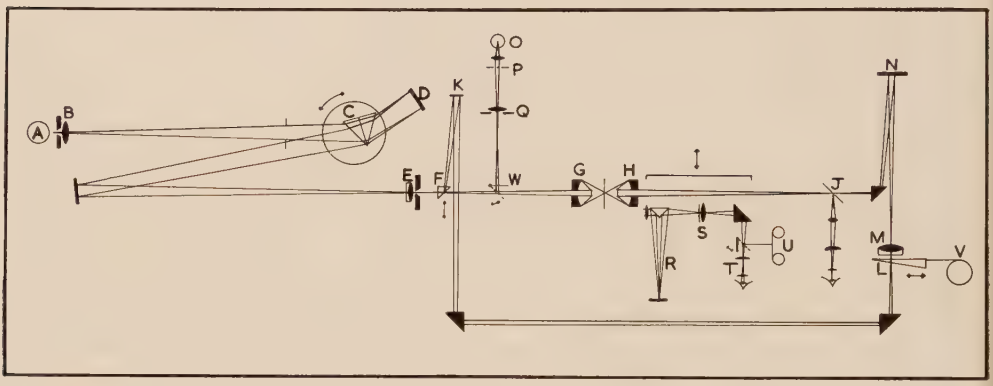

Fig. 1. Optical paths of recording microspectrophotometer.

A High pressure Xenon Arc (Osram XBO 162)
B Field lens
C Cornu prism
D Concave mirror
E Exit slit
F Beam alternator (moving prism)
G Condenser
H Objective
J Stainless steel mirror with central aperture
K Concave mirror
L Optical attenuator
M Achromatic lens
N Photocathode
O 125w Mercury Arc (B.T.H. type MB/D)
P Phase annulus
Q Annulus imaging lens and visible field stop
R Phase contrast imaging system
S Phase retardation plate
T Eyepiece
U camera
V Servo-motor
W Removeable 45° mirror
X Recording chart

In this preliminary communication we will describe briefly the instument with reference to Figs. 1 and 2. The source, supplied by direct current, is normally a 160 watt high pressure Xenon arc (*A*) (Osram XBO 162) which illuminates the input slit and field lens (*B*) of the monochromator. This, in turn, is focused by the concave mirror (*D*), behind the cornu prism (*C*), on to the exit slit (*E*), which is also the field stop of the microscope. The input slit of the monochromator is automatically adjusted to maintain a nominal 2 mμ bandwidth at the fixed output slit. A beam alternating device (*F*) is placed immediately behind the field stop and consists of a small 45° quartz prism mounted on a hardened slide running on balls. The prism is vibrated at 50 cycles per second by a synchronous motor so that the monochromated radiation alternately passes direct to the microscope (*G*, *H*) or is reflected to form the reference beam (*K*, *L*). The microscope is a conventional arrangement of condenser (*G*) and objective (*H*) together with an auxiliary micro-

scope and 45° mirror (*J*) for viewing the limiting aperture in front of the photomultiplier. The reference beam optics consist only of a concave mirror (*K*) which forms a magnified image of part of the field stop on to the attenuator slit (*L*) and a quartz-lithium fluoride achromatic lens (*M*) above the attenuator, which in turn forms an image of the concave mirror (*K*) on the

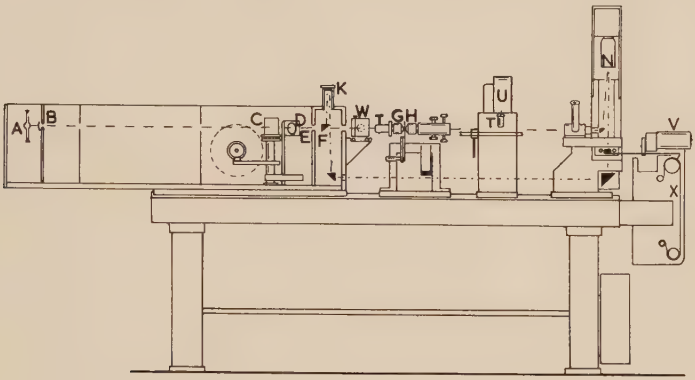

Fig. 2. Side view (semi-diagrammatic) of the recording microspectrophotometer, lettered as in Fig. 1.

photomultiplier cathode (*N*). In addition to these simple components required during recording, there are the auxiliary light train (*O*, *P*, *Q*, *W*) and the components (*R*, *S*, *T*) which are needed for viewing and photographing objects with green phase-contrast illumination introduced by the mirror (*W*). The images in both viewing systems (*J* and *T*) are parfocal and centered to each other.

The attenuator itself consists of an aperture of logarithmic form designed to give a linear scale of density (0–1.0) and was made by etching copper with a photo-mechanical process from a negative reduced from a master-form. The uniformity of the limiting slit at the attenuator may be checked at any time with the aid of an oscilloscope. This attenuator replaces the slide wire and slider of the potentiometer recorder mounted at the end of the instrument so that the pen records the position of the attenuator on the chart roll (*X*).

The paper is moved by a second servo-mechanism and may be electrically coupled to either (a) the wavelength drum of the monochromator for recording absorption spectra, or (b) the hydraulic system moving the quartz-plate precision stage (see Caspersson) for scanning cells at a single wavelength, or (c) a synchronous motor. This servo-system uses 6″ diameter A.C. potentiometers of precision design to generate the necessary input voltages.

The main problem in the design of the amplifier is to maintain the loop-

gain of the servo system at an approximately constant value for all light levels, since the performance of the mechanism would otherwise be seriously impaired. Ideally this means that the servo-amplifier should possess a logarithmic characteristic, but in practice it has been found adequate and more convenient to use the power law characteristic of the substance "Metrosil".

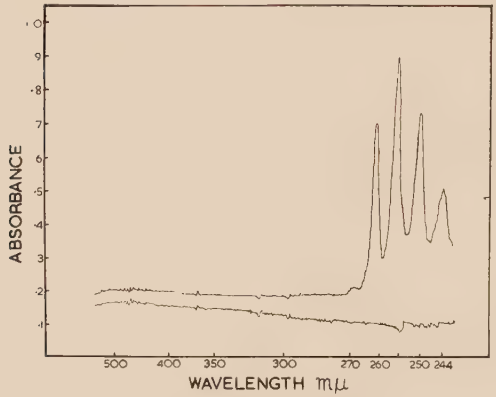

Fig. 3*a*. Absorption spectrum of benzene diluted with cyclohexane. Background trace displaced to show similarities.

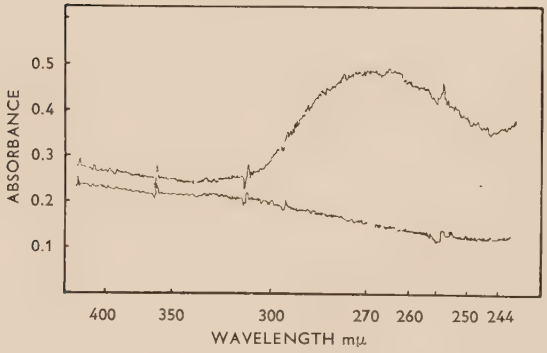

Fig. 3*b*. Absorption spectrum of a living tissue culture nucleus.

When a "Metrosil" element is used as the anode load of the photomultiplier the loop gain changes by a factor of about 5 as the signal current in the photo multiplier varies over a range of 700 -1. This change in loop-gain is insufficient to affect adversely the performance. The remainder of the amplifier is of conventional A.C. design and the servo-mechanism is stabilised by a fedback voltage provided by a generator coupled to the servo-motor (*V*).

The two optical paths are dissimilar and therefore the trace recorded from a non-absorbing specimen will not be linear owing to differences in the transmission of the two beams. Fortunately the solid quartz objectives have a high transmission in the ultraviolet and the deviation from non-linearity

is only about 0.15 density units, and in many applications for which the apparatus was designed the medium, and therefore the background trace, will have considerable absorption. Typical traces of (a) benzene and (b) a living tissue culture "nucleus" are shown in Fig. 3. The noise level on the traces varies from 0.1 per cent at 450 mμ to 0.4 per cent at 245 mμ, with an aperture corresponding to 2μ diameter on the specimen and a nominal spectral bandwidth of 2 mμ. Much of the detail occurring in each of the pair of traces is due to the line structure superimposed on the continuum of the Xenon arc (especially in the 450–500 mμ region), and to the difficulty of selecting exactly the same part of the monochromator exit slit for illuminating both beams.

It will be seen from Fig. 2 that the whole instrument is mounted as a single unit, which together with the method of attenuation employed, gives great mechanical and electrical stability. Measurements on small test objects, and tests for objective glare, monochromator scattered light and linearity of the attenuator have all proved satisfactory. (It is hoped to publish a fuller account of these together with details of the design at a later date.)

We would like to thank Professor J. T. Randall, F.R.S. for supporting this project, and all the members of the workshop staff who have helped to make the microspectrophotometer. One of us (E. M. D.) is indebted to the Nuffield Foundation for the award of a Research Fellowship.

"Metrosil" elements were kindly supplied by Mr. N. L. Constantine, Metropolitan-Vickers Electrical Company Limited.

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PÉNÉTRATION DU BENZIMIDAZOLE DANS LA CELLULE DE LEVURE

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L'étude de l'inhibition par le benzimidazole (BZI) de la multiplication cellulaire de la levure [12] et de la synthèse des enzymes respiratoires par les cellules non-proliférantes [13] a montré que seule la molécule non ionisée du BZI est biologiquement active. Pour expliquer cette constatation on a émis l'hypothèse que la levure serait imperméable à la molécule de l'inhibiteur chargée positivement. Le présent travail est destiné à soumettre cette hypothèse à la vérification expérimentale directe qui consiste à mesurer les quantités de BZI intracellulaire et extracellulaire en fonction de la charge de la molécule.

MATÉRIEL ET MÉTHODES

Les souches de levure utilisées et leurs conditions de culture ont été les mêmes que celles utilisées précédemment [12–14].

Dosage du BZI.—Le dosage est fondé sur le spectre d'absorption du BZI (produit BDH, Londres) dans la région de 230 à 280 mμ. Il est effectué au spectrophotomètre Beckman, modèle DU. Comme on le voit sur la Fig. 1, le spectre varie en fonction du pH, ce qui est dû à la dissociation ionique du groupe imidazole de la molécule. Les variations du spectre permettent de calculer la constante de dissociation apparente [cf. 11]. La valeur trouvée est sensiblement la même (5,45) que celle mesurée par l'électrotitration. Les points isosbéstiques sont : 253 mμ, 269 mμ, 272 mμ, et 275 mμ. Les valeurs des ε mM des maxima d'absorption de la forme ionisée sont : 3,76 à 241 mμ, 6,00 à 266 mμ et 5,39 à 273 mμ; les valeurs correspondantes pour la forme non ionisée sont 5,16, 3,72 et 3,52 respectivement.

Dans les expériences destinées à mesurer la disparition d'une substance du fait de sa pénétration dans les cellules on est obligé d'avoir recours à des suspensions cellulaires très denses (20 à 50 % du volume total étant représenté par le volume des cellules). Les liquides surnageants obtenus par centrifugation de ces suspensions denses de levure montrent souvent une absorption avec un maximum vers 260 mμ qui pourrait être attribuée aux acides nucléiques. Ce fait pourrait introduire une erreur considérable dans

le dosage du BZI car la grandeur de cette absorption ne peut pas être estimée directement. La mesure de l'absorption sur une partie aliquote de la suspension sans BZI, suivie de la soustraction des valeurs obtenues, ne peut pas être utilisée. Etant donnée, en effet, l'action du BZI sur le métabolisme général de la levure [12–15], il est clair qu'il pourrait influencer l'excrétion des substances responsables de cette absorption. Le procédé qui permet d'éviter cette difficulté utilise les variations du spectre du BZI en fonction du pH, le fait que son spectre présente plusieurs maxima et le fait que l'absorption de base ne varie que relativement peu avec le pH. On calcule la concentration du BZI d'après les mesures faites sur des prises aliquotes amenées aux pH 2 et 8 selon la formule :

$$\text{BZI (m}M\text{)} = \frac{E \text{ total à pH } 2 - E \text{ total à pH } 8}{\varepsilon\,\text{m}M \text{ total du BZI à pH } 2 - \varepsilon\,\text{m}M \text{ du BZI à pH } 8}$$

La concordance des valeurs obtenues à des maxima et des minima différents du spectre prouve la validité du procédé.

Mesure de la pénétration.—Nous allous exprimer la pénétration du BZI (total = ionisé + non ionisé) par la formule qu'on obtient à partir de l'équilibre de diffusion à travers la membrane :

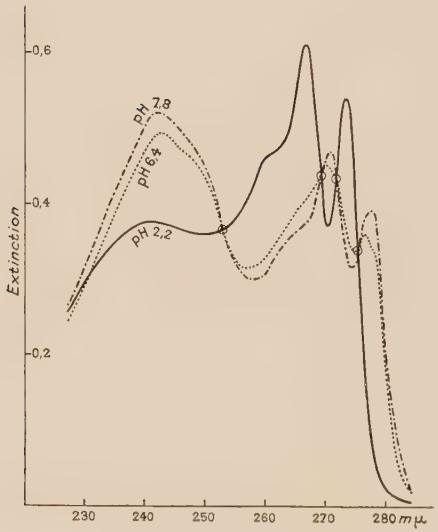
$$R = \frac{V_0}{V_c}\left(\frac{C_0}{C_m} - 1\right) + \left(1 - \frac{V_m - V_0}{V_c}\right)$$

où R = coefficient de répartition entre la cellule et le milieu extérieur,
V_0 = volume de la solution du BZI avant l'addition de cellules,
C_0 = concentration du BZI dans la solution avant l'addition de cellules,
V_m = volume de la suspension après le mélange de cellules et du BZI,
C_m = concentration du BZI restant dans la solution après le mélange des cellules et du BZI,
V_c = volume des cellules.

Dans cette expression les valeurs de V_0, V_m, C_0 et C_m peuvent être déterminées directement avec une bonne précision. Le volume cellulaire (V_c) est déterminé en mesurant le volume du culot après une centrifugation à froid de 15 min. à 2000 G et en soustrayant 22 % du volume du culot pour l'eau interstitielle [cf. 3]. Dans les conditions de l'expérience présentée par la Fig. 2, nous avous par exemple trouvé que le volume d'un gramme (poids sec) de cellules était de $2{,}9 \pm 0{,}1$ cm³. Cette valeur n'est pas différente de celle déterminée avec beaucoup plus de précision pour d'autres levures [2, 9]. Il est d'ailleurs à noter que dans les conditions de cette expérience

l'erreur sur l'estimation de V_c ne se répercute que faiblement sur les valeurs de R, supérieures à l'unité.

L'expression permettant de calculer R n'est valable que si le corps étudié n'est pas métabolisé par la cellule. Comme on le verra plus loin, ceci est le cas pour le BZI, car on peut récupérer la presque totalité du corps absorbé.



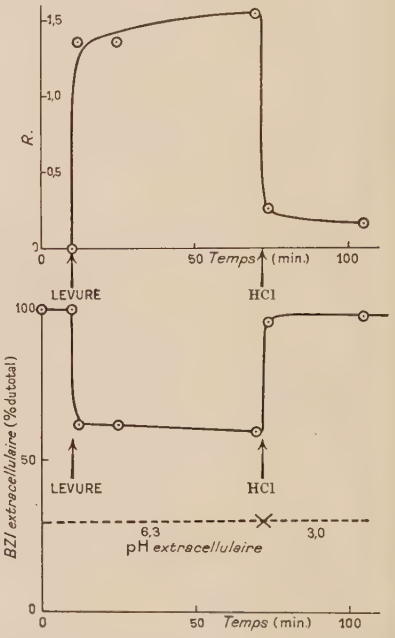


Fig. 1. Spectre d'absorption du BZI 0,1 mM en fonction du pH.
Fig. 2. Pénétration du BZI dans la cellule et son expulsion à la suite de l'abaissement du pH extracellulaire.

La formule est générale et s'applique aussi bien à des suspensions de cellules qu'à la levure pressée avec très peu d'eau interstitielle. Dans ce deuxième cas on arrive à une expression plus simple, analogue à celle utilisée par Conway et Downey [3]. Le coefficient R peut *a priori* être égal à 0, compris entre 0 et 1 ou plus grand que l'unité. Dans le premier cas la cellule est totalement imperméable, dans le deuxième la pénétration (ou l'absorption) n'est que partielle et dans le troisième la cellule concentre le corps par rapport au milieu environnant.[1]

[1] Le coefficient R tel qu'il est défini dans ce travail exprime la répartition entre la cellule et le milieu extérieur. En réalité, la diffusion du BZI a lieu entre l'eau intracellulaire et l'eau du milieu extérieur. Le rapport entre le volume de l'eau intracellulaire et le volume cellulaire total est, chez la levure, de l'ordre de 0,7 [cf. 2]. Par conséquent il est vraisemblable que la cellule concentre le

RÉSULTATS EXPÉRIMENTAUX

Dans le premier type d'expérience, on mélange la levure de boulangerie pressée (marque « La Parisienne » prise à la sortie de la levurerie) avec du BZI et on dose le BZI restant en solution après l'établissement de l'équilibre. Le tableau I donne les résultats d'une expérience effectuée à froid où le V_0 = 3,0 cm³ et la durée de contact est d'une heure. On voit qu'à pH 8,3, où plus de 99 % du BZI extracellulaire se trouve sous forme non ionisée, la quantité de BZI disparue est très grande et proportionnelle au volume de cellules ajoutées. A pH 2,8, où presque tout le BZI extracellulaire est ionisé, la quantité disparue est au contraire très faible. Dans ces dernières conditions, le coefficient R ne peut être estimé qu'avec très peu de précision. En effet, la teneur en BZI intracellulaire étant déduite de la diminution du BZI restant en solution, une erreur dans le dosage spectrophotométrique de 5 % peut entraîner une différence de 100 % dans l'estimation de R. On peut conclure de cette expérience que le « volume cellulaire perméable » à la forme ionisée du BZI est de l'ordre de 30 % du volume cellulaire total ($R \cong 0{,}3$), tandis que le « volume cellulaire perméable » à la molécule non ionisée est dix fois environ plus grand ($R \cong 2{,}6$).

Le deuxième type d'expérience est destiné à étudier la cinétique de la pénétration de la forme non ionisée du BZI et l'effet produit par l'abaissement du pH extracellulaire sur le BZI absorbé par la cellule. Les résultats d'une expérience effectuée parallèlement et dans les mêmes conditions que celles utilisées pour la mesure de l'adaptation respiratoire [13, 14] sont donnés par la fig. 2. On mélange 20,2 cm³ de cellules (souche « Yeast Foam », cultivée en anaérobiose), contenus dans 60 cm³ de tampon phosphate 0,2M pH 6,3, avec 30 cm³ de BZI 2,8 mM dissous dans le même tampon. Le mélange est incubé en aérobiose à 28°C. Après 2, 13 et 60 minutes on prélève des prises aliquotes qui sont centrifugées immédiatement à froid pendant 10 min. et servent à doser le BZI restant en solution. A la 62e minute on acidifie le mélange jusqu'à pH 3,0 par addition de HCl, puis on effectue des prélèvements. Comme on le voit sur la figure 2, la pénétration à pH 6,3 est très rapide et l'équilibre est atteint en quelques minutes. Le coefficient R est de 1,5. Aussitôt après l'acidification qui entraîne l'ionisation du BZI extracellulaire, la presque totalité du BZI préalablement absorbé sort des cellules et on retrouve dans le surnageant 97 % du corps initialement présent. Le coefficient R à pH 3,0 est de l'ordre de 0,2.

BZI par rapport au milieu environnant, non seulement lorsque la valeur de R dépasse l'unité, mais déjà lorsqu'elle est supérieure à 0,7.

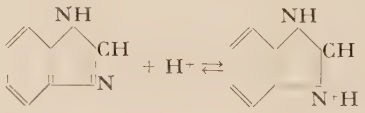
TABLEAU I

Pénétration du BZI dans la levure à pH 2,8 et à pH 8,3.

pH extracellulaire	Volume des cellules (cm^3)	BZI extracellulaire		BZI absorbé (% du total)	R
		initial (mM)	final (mM)		
2.8	0,88	0,800	0,758	5,3	0,19
	0,93	»	0,719	10,1	0,48
	1,43	»	0,696	13,0	0,29
	1,77	»	0,701	12,3	0,19
	1,80	»	0,678	15,2	0,30
	moyenne	—	—	—	$0,29 \pm 0,2$
8,3	0,92	»	0,483	39,6	2,1
	0,97	»	0,419	47,7	2,8
	1,45	»	0,352	56,0	2,6
	1,70	»	0,306	61,7	2,8
	1,81	»	0,310	61,3	2,5
	1,89	»	0,308	61,5	2,5
	moyenne	—	—	—	$2,6 \pm 0,5$

DISCUSSION

Le benzimidazole (1,3 benzodiazole) est une base faible dont la première dissociation peut être représentée comme suit :



La constante apparente de cette dissociation basique (pK'_{A_1}) a été déterminée à plusieurs reprises et trouvée égale à 5,3–5,4 [10, 13, 14, 16, 17]. La constante de la deuxième dissociation correspond à une dissociation acide du groupe —NH—. Du fait de sa valeur élevée [$pK'_{A_2} = 12,3$ (16)]cette ionisation n'intervient pas dans la région des pH compatibles avec la survie de la levure et utilisés dans le présent travail.

Nos résultats montrent que la levure n'est que faiblement perméable à l'ion BZI^+. Le « volume cellulaire perméable » est de l'ordre de $20\% \pm 10\%$ du volume cellulaire total. Il est très probable que ce chiffre (déterminé avec peu de précision pour les raisons exposées plus haut) représente la pénétration du BZI^+ dans la « région extérieure », [3], de la cellule de levure

TABLEAU II

Répartition théorique et trouvée du benzimidazole dans le milieu et dans la cellule.

Milieu			Cellule					*R*	
			Paroi		Intérieur				
pH	(BZI)	(BZI^+)	(BZI)	(BZI^+)	pH	(BZI)	(BZI^+)	Théorique	Trouvé
8,3	1,00	<0,01	1,00	<0,01	5,8	1,00	0,38	1,28	2,6 ±0.5
6,3	0,90	0,10	0,90	0,10	5,8	0,90	0,28	1,16	1,5 ±0,2
3,0	<0,01	1,00	<0,01	1,00	5,8	<0,01	<0,01	0,11	0,15±0,1

étudiée par Conway et Downey. Ces auteurs interprètent la « région extérieure » comme étant la paroi cellulaire, très épaisse chez la levure. Plusieurs ions et acides organiques y pénètrent librement. Ils sont arrêtés là par la membrane cytoplasmique interne qui est beaucoup plus sélective et ne laisse pas passer les ions. Si nous admettons que le BZI^+ ne pénètre que dans la paroi cellulaire, nous pouvons conclure que *les processus qu'il inhibe* [12, 13, 14, 15] *ont lieu à l'intérieur de la cellule et non dans, ou sur, la paroi.* Il n'exerce, en effet, aucune action inhibitrice lorsqu'il a pénétré en tant qu'ion.

La molécule non ionisée pénètre librement à l'intérieur de la cellule et *la valeur de R peut dépasser considerablement l'unité.* Deux explications peuvent rendre compte de ce fait. Elles sont fondées toutes les deux sur la possibilité qu'une fraction des molécules intracellulaires soit soustraite de façon à ce qu'elles ne participent plus à l'équilibre de diffusion à travers la membrane. Soit qu'il s'agisse d'une ionisation intracellulaire, soit qu'ait lieu une fixation par les récepteurs intracellulaires :

Etant donnés le pH intracellulaire [cf. 4, 8] et le volume de la « région extérieure » [cf. 3] perméable aux ions BZI^+, il est facile de calculer la valeur de *R* qu'on devrait trouver à des pH extracellulaires différents. Le tableau II donne la comparaison des valeurs calculées et trouvées expérimentalement. Les *R* expérimentaux sont plus élevés que ceux prévus théoriquement. Autrement dit, une fraction plus grande des molécules pénétrantes (c'est-à-dire non chargées) est soustraite de l'équilibre de diffusion que celle résul-

tant de l'ionisation intracellulaire. Ceci peut évidemment être dû à une combinaison de BZI avec les récepteurs intracellulaires. Comme on voit dans le même tableau, l'expulsion, lors de l'abaissement du pH extracellulaire (cf. Fig. 2), du BZI préalablement absorbé, s'explique clairement. Elle résulte de l'imperméabilité de la mebrane à l'ion BZI^+ et du maintien du pH intracellulaire. Dans ces conditions, les valeurs de R prévues et trouvées s'accordent de façon satisfaisante.

On connaît de très nombreux exemples dans lesquels l'activité biologique est due à la charge ou à l'absence de charge d'une molécule [cf. 1]. On connaît également de nombreux exemples de perméabilité sélective des membranes cellulaires envers des molécules non chargées et les ions correspondants [cf. 5, 6, 7]. Dans le cas du benzimidazole, il a été possible de montrer par des mesures directes que l'ion n'est pas actif car il ne pénètre pas à l'intérieur de la cellule de levure.

RÉSUMÉ

1) Une méthode de dosage du benzimidazole (BZI), fondée sur le spectre d'absorption, est décrite et utilisée pour la mesure de sa pénétration dans la cellule de levure.

2) L'intérieur de la cellule n'est perméable qu'à la molécule non chargée (pK'_{A_1} 5,3). Le volume perméable à l'ion BZI^+ ne représente que 10 à 20 % du volume cellulaire total.

3) La concentration en BZI intracellulaire est supérieure à celle prévue théoriquement par l'équilibre de diffusion à travers la membrane imperméable à l'ion BZI^+. Une fraction du BZI semble être combinée par des récepteurs intracellulaires et soustraite ainsi de l'équilibre de diffusion. Lors de l'abaissement du pH extracellulaire, le BZI préablement absorbé diffuse rapidement en dehors de la cellule.

4) Ces faits permettent d'expliquer l'absence de l'activité biologique de l'ion BZI^+, ainsi que la réversion de l'inhibition provoquée par l'abaissement du pH extracellulaire.

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STUDIES ON THE ACROSOME

V. AN EGG-MEMBRANE LYSIN FROM THE ACROSOMES OF *MYTILUS EDULIS* SPERMATOZOA

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Investigations concerned with lytic substances of spermatozoa were given their initial impulse by J. Loeb's theory of the mechanism of fertilization, which postulated a sperm-borne cytoplasmic lysin together with a "corrective factor".

Following the work of Hibbard [6], who found a substance in amphibian sperm extracts which dissolved the chorionic layers of the unfertilized egg, several European workers [16, 17, 4, 10] believed that they had demonstrated the presence of an egg-activating "enzyme" such as that required by Loeb's theory. Experiments purporting to prove its localization in the acrosome (of spermatids), however, were less than convincing and have not been confirmed.

Runnström and his co-workers [11, 12] have also obtained from sea urchin spermatozoa, by a rather drastic extraction procedure, a methanol-soluble substance, described as an unsaturated fatty acid, which they can show to have a softening effect on the surface layers of unfertilized eggs. They have not yet demonstrated this substance to be present in effective amounts under physiological conditions.

An entirely different sort of lysin, specific for the metaplasmic "egg-membranes" or vitelline membranes of molluscan eggs, and clearly having no effect on the underlying cytoplasm, was discovered by Tyler [13] in extracts of the spermatozoa of the gastropod, *Megathura crenulata*. Krauss [7], extending Tyler's study, found the lysin to be released in considerable quantities from the living spermatozoa at alkaline pH (9.5), and further determined

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[8] that a similar lysin, capable of dissolving preserved *Megathura* egg-membranes, is contained in extracts of sea urchin spermatozoa. Berg [1, 2] also demonstrated such a lysin in extracts of frozen-thawed spermatozoa of the mussel, *Mytilus edulis*.

In the course of a study of the acrosome reaction in various bivalve molluscs [3], it was found that in at least twelve species scattered through this class, the spermatozoan acrosome breaks down, normally on contact with the egg surface, leaving only a very slender filament in the place of the often conspicuous acrosome. Among the spermatozoa studied, that of *Mytilus edulis* was found to have an exceptionally prominent acrosome, about 3.5μ in length. Since the dissolution of such a large structure might be expected to release a fairly easily detectable quantity of substance into the medium, these spermatozoa were selected as material for an attempt to determine what part of the spermatozoan contains the egg membrane lysin.

MATERIALS AND METHODS

All the experiments were performed with the gametes of *Mytilus edulis*, obtained from animals collected periodically in the immediate vicinity of the Misaki Station, and kept in live-cars until just before use. Reactive gametes were secured from the middle of October to the latter part of April.

The details of the procedure for inducing spawning by electrical stimulation were reported in the previous paper [3]. When spermatozoa are shed "dry" or into sea water, however, a rather high percentage of them (20–25 per cent) spontaneously undergo the acrosome reaction. In order to obtain dense suspensions containing a minimum of reacted spermatozoa, the sperm-containing mantles of electrically stimulated males were extirpated and placed on a piece of cheesecloth in about 5 cc of Ca-free artificial sea water. The mantle tissue and mucus are caught in the cheesecloth, while the spermatozoa swim out into the medium, so that a relatively clean suspension results. The percentages of reacted sperm in such suspensions are somewhat lower than in those obtained by shedding.

If *Mytilus* egg-water is added to a sperm suspension in sea water, about 50 per cent of the spermatozoa will usually show the typical acrosome reaction (Figs. 1, 2), with complete disappearance of the whole acrosome except the slender acrosome filament. This method, however, gives only about a 25 per cent increase of reacted sperm over the original suspension, besides introducing the unknown quantities of the dissolved jelly and other substances making up the "egg-water", and involving a considerable dilution.

On the other hand, it was found that the addition of about 10 per cent by volume of 0.36M $CaCl_2$ to the sea water suspension, and about 15 per cent to the Ca-free suspension, causes between 90 and 95 per cent of the spermatozoa to undergo an acrosome reaction. This may be either the typical reaction, or a somewhat atypical one, in which the distal, external part of the acrosome breaks down

completely, and the proximal part to a considerable extent, leaving a filament which is shorter and apparently stouter than the normally extruded one, as well as a certain amount of amorphous material at the base of the acrosome. On the assumption that the rapidly-dissolving portion of the acrosome represents the lytic material, this method was employed to induce the acrosome reaction in the mass suspensions used in the experiments.

Equally high percentages of the same sort of reaction were found to occur in sperm suspensions which had been exposed to low temperature (2–4° C) for several hours. In both cases the reacted spermatozoa (with acrosomes broken down) are normally active and show no other morphological changes, although their movement is clearly less vigorous than that of the unreacted spermatozoa in the same suspension.

In order to dermine the percentages of reacted spermatozoa, samples of the suspensions were killed by the addition of a small amount of a chromic acid-OsO_4 mixture, and suitably diluted subsamples were counted under the microscope. Usually 200 spermatozoa were counted in each of four subsamples.

The lysin was obtained by centrifuging the suspensions first at about 600 *g* for 10 minutes to remove the greater portion of the spermatozoa with a minimum of injury, and then at 2300 *g* for about 30 minutes. There were always a few intact spermatozoa as well as other small particles left in the supernatants. However, tests on supernatants rendered completely particle-free (phase-contrast, oil immersion observation) by centrifuging at 60 lb pressure with an air-turbine centrifuge showed them to possess full lytic activity, indicating that the lysin is present in solution in the sea water supernatant.

In spite of the precautions which were taken in centrifuging the concentrated sperm suspensions to prevent injury to the cells, there were always a few tails found in the supernatants after the second, stronger centrifugation, indicating that a small proportion of the sperm heads had been disrupted in the handling process. As check against the possibility that the lysin might be contained in some other part (besides the acrosome) of such cells, suspensions to which excess Ca^{++} had been added to break down the acrosomes were left standing undisturbed in test tubes at 2–3° C for about 24 hours. Under these conditions the spermatozoa settled out of suspension to some extent, leaving a practically sperm-free supernatant, which showed strong lytic activity. The spermatozoa in such suspensions were normally active on return to room temperature and no disrupted cells or any other indication of injury to the spermatozoa could be detected. Control suspensions of unreacted sperm kept under the same conditions retained apparently full fertilizing capacity.

Extracts of intact and reacted spermatozoa were made by grinding frozen-thawed suspensions in a glass mortar with sand, extracting with sea water and centrifuging until a relatively debris-free supernatant was obtained.

In order to assay the lytic activity of the lysin solutions and sperm extracts thus prepared, it is desirable, as Berg [2] pointed out, to test them on unfertilized eggs which have been plasmolyzed to separate the membrane from the normally closely adhering cytoplasmic surface. Since, however, it was found that the membranes of eggs which were left standing in hypertonic (e. g., 2 l.c. concentrated) sea water alone tended to swell and disintegrate, the following standard procedure was adopted. Single drops of a concentrated suspension of fresh eggs are placed separately on a dry Syracuse watch glass and left uncovered until the water has evaporated com-

pletely. By this method the eggs are plasmolyzed, and the further evaporation of the sea water prevents any disintegrating effect on the membranes. When a sample of lysin is to be tested, the eggs from one of the evaporated egg drops are resuspended in sea water and transferred to a slide glass, where the lysin is added in known proportions.

RESULTS

Localization of lysin in acrosome.—Reciprocal series of experiments were done to demonstrate (1) the presence of lysin in the supernatants from reacted sperm suspensions and its absence in extracts of these same spermatozoa, and (2) the absence of lysin in the supernatants from unreacted sperm suspensions and its presence in extracts of the spermatozoa. In a third series, the relative lytic activity of the supernatant was plotted against the percentage of reacted sperm in a number of similar suspensions.

In view of the difficulty of determining accurately the concentrations of such dense sperm suspensions, added to the inevitable variability of other factors in studies involving gametes, it was decided to treat each experiment as a separate unit. The data in Table I must therefore be read *only horizontally*, as indicating the relative lytic activity of each supernatant as compared with the lytic activity of the extract made of the spermatozoa removed from that particular suspension and diluted to the same extent.

TABLE I

Comparison of lytic activities of supernatants and sperm extracts in reacted and unreacted sperm suspensions.

Experiment No.	Per cent reacted	Time (min.) required to dissolve vitelline membranes of plasmolyzed eggs	
		Supernatant	Sperm extract
Series (1)			
Exp. 6	90	2	51
Exp. 13 . . .	95	0.5	30
Exp. 14 . . .	92	1.7	30
Series (2)			
Exp. 15 . . .	14	80	2
Exp. 16 . . .	15	13	2
Exp. 17 . . .	11	35	1.5

Series (1): acrosome material mostly in supernatant.
Series (2): acrosome material mostly in sperm extract.

In the three experiments shown in Series (1), sea water was added to shed "dry" sperm to give approximately 10 per cent suspensions, since it was found that the percentages of cells reacting to added Ca^{++} were lower in more concentrated suspensions. After a very light centrifuging to remove large cellular debris, excess Ca^{++} was added to induce acrosomal breakdown, and samples were fixed for counting. The spermatozoa were centrifuged out and frozen, and the supernatants were further centrifuged, removing all but an insignificant number of the remaining spermatozoa. The cleaned supernatants were then stored at 2–3° C during the preparation of sea water extracts of the frozen-thawed reacted sperm. The paired solutions were finally tested alternately for membrane lysis, using eggs from the same female which had been plasmolyzed simultaneously. Plasmolyzed egg suspensions and test solutions were mixed in equal amounts.

The test for lytic activity consisted in measurement of the time required for at least a certain definite degree of swelling and beginning of disintegration of the membrane in roughly half the plasmolyzed eggs under observation (Figs. 3, 4). There is admittedly a larger error involved in such measurement, since strong preparations of lysin dissolved the membranes completely almost before they could be observed properly, while the gradual swelling and dissolution found in very weak lysin solutions may have been due in part or wholly to the action of the hypertonic sea water.

Table I, Series (2) gives the results of three of the reciprocal experiments, in which the spermatozoa were collected in Ca-free sea water to reduce the percentages of spontaneous reaction. After samples had been fixed for counting, the spermatozoa were carefully centrifuged out and frozen, and the supernatant and sperm extract prepared and tested as in the previous series.

Because of the various sources of error in these experiments, small differences in the data are of no significance. The results do, however, indicate clearly that the lytic activity of the *Mytilus* spermatozoan is associated with the material of the acrosome.

This relation also appears in the results from a number of tests which were made of the lytic activity of supernatants from similar sperm suspensions in which the acrosome reaction had occurred in various percentages following the addition of different amounts of excess Ca^{++} (Fig. 5). In arranging these data, the lytic activities found in the supernatants of suspensions which had reacted maximally (i.e. 85–95 per cent) were taken as 1.0, and the other values expressed in percentages of this figure. Again it must be emphasized that the points appearing on the graph represent only a tendency, but considered

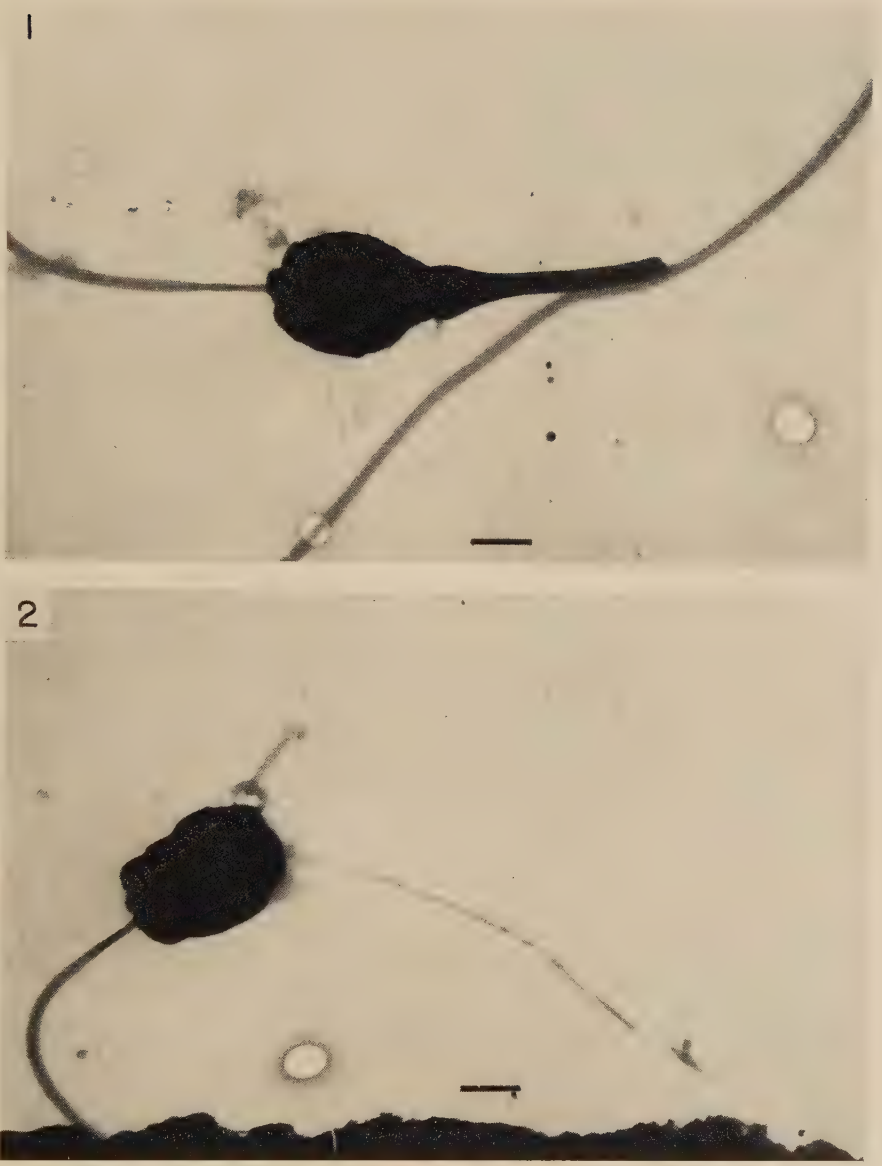


Fig. 1. Electron micrograph of *Mytilus edulis* spermatozoan, fixed with chilled neutral formalin. From DAN and WADA, 1955. Courtesy of the *Biological Bulletin*.
Fig. 2. Electron micrograph of *Mytilus* spermatozoan after acrosome reaction. From DAN and WADA, 1955. Courtesy of the *Biological Bulletin*.

together they undoubtedly indicate that the lytic activity found in the supernatants of *Mytilus* sperm suspensions is directly proportional to the number of sperm in which the acrosome reaction has taken place.

Role of acrosome lysin in fertilization.—The *Mytilus* egg is surrounded by a layer of jelly (Fig. 6) which is apparently not dispersed by even prolonged exposure to strong lysin solutions (Fig. 7). The surface layers of the unfer-

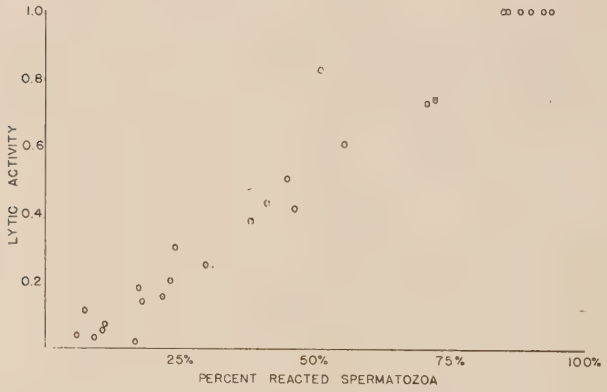


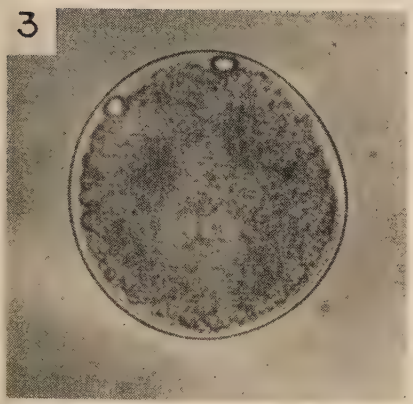
Fig. 5. Activity of lysin-containing supernatant plotted against percentage of reacted spermatozoa. Approximately equivalent sperm suspensions were divided into several lots, which were treated so that the acrosome reaction occurred in varying degrees. The highest activity was taken as 1.0, and the lower activities expressed in percentages of this.

tilized egg consist of an extremely thin vitelline membrane[1] and an underlying hyaline layer, the greater part at least of which adheres to the vitelline membrane when the cytoplasmic surface shrinks in hypertonic solutions. There is no immediate change in the appearance of these layers at fertilization.

When normal fertilized eggs are exposed to lysin solutions, the vitelline membrane is dissolved by exposures comparable to those required to remove the membrane from plasmolyzed eggs, but the hyaline layer does not visibly diminish in thickness until after much longer exposures. The absence of

[1] Field [5] has reported, "A distinct vitelline membrane no less than 1μ in thickness envelops the [*Mytilus*] egg" (p. 187). There is little doubt that he included the width of the hyaline layer in this estimate. In this connection, the reader is referred to Fig. 12, in which one of Meves' sections shows the vitelline membrane separated from the hyaline layer as a result of fixation.

Fig. 3. Egg of *Mytilus*, plasmolyzed by evaporation and resuspended in sea water.
Fig. 4. Plasmolyzed egg in acrosome lysin solution; membrane swollen and partly dissolved.
Fig. 6. Unfertilized *Mytilus* egg in India ink suspension, showing jelly layer ca 8μ wide surrounding egg.
Fig. 7. *Mytilus* 2-cell stage in India ink. Egg was exposed to moderately strong lysin solution for 90 minutes, as evidenced by separation of blastomeres, but jelly layer has not been dispersed by action of lysin.
Fig. 8. Phase contrast photograph of *Mytilus* egg in sea water; polar bodies are compressed under membrane layers.
Fig. 9. *Mytilus* egg in lysin solution; polar bodies are free from egg surface (phase contrast).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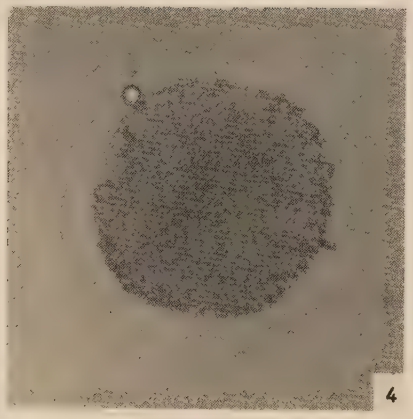
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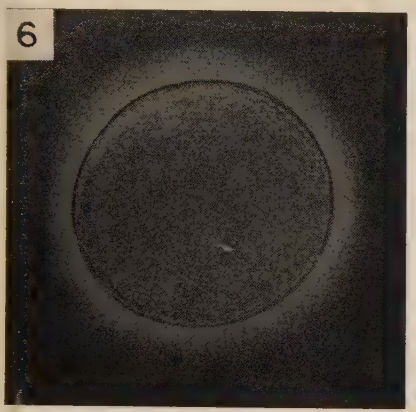
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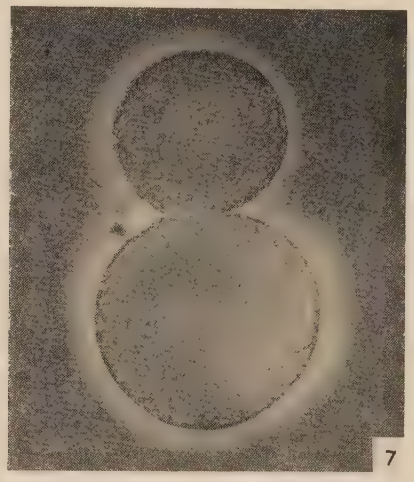
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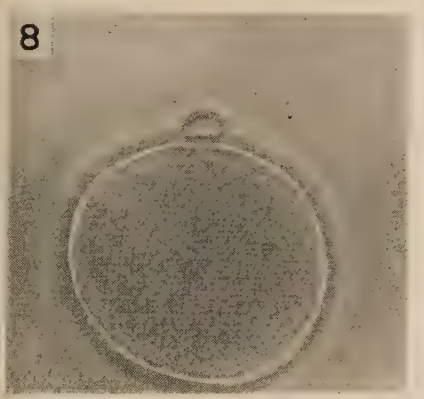
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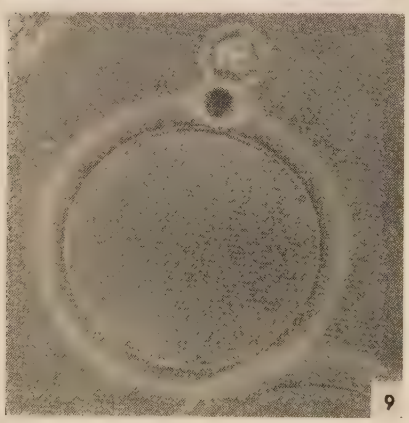
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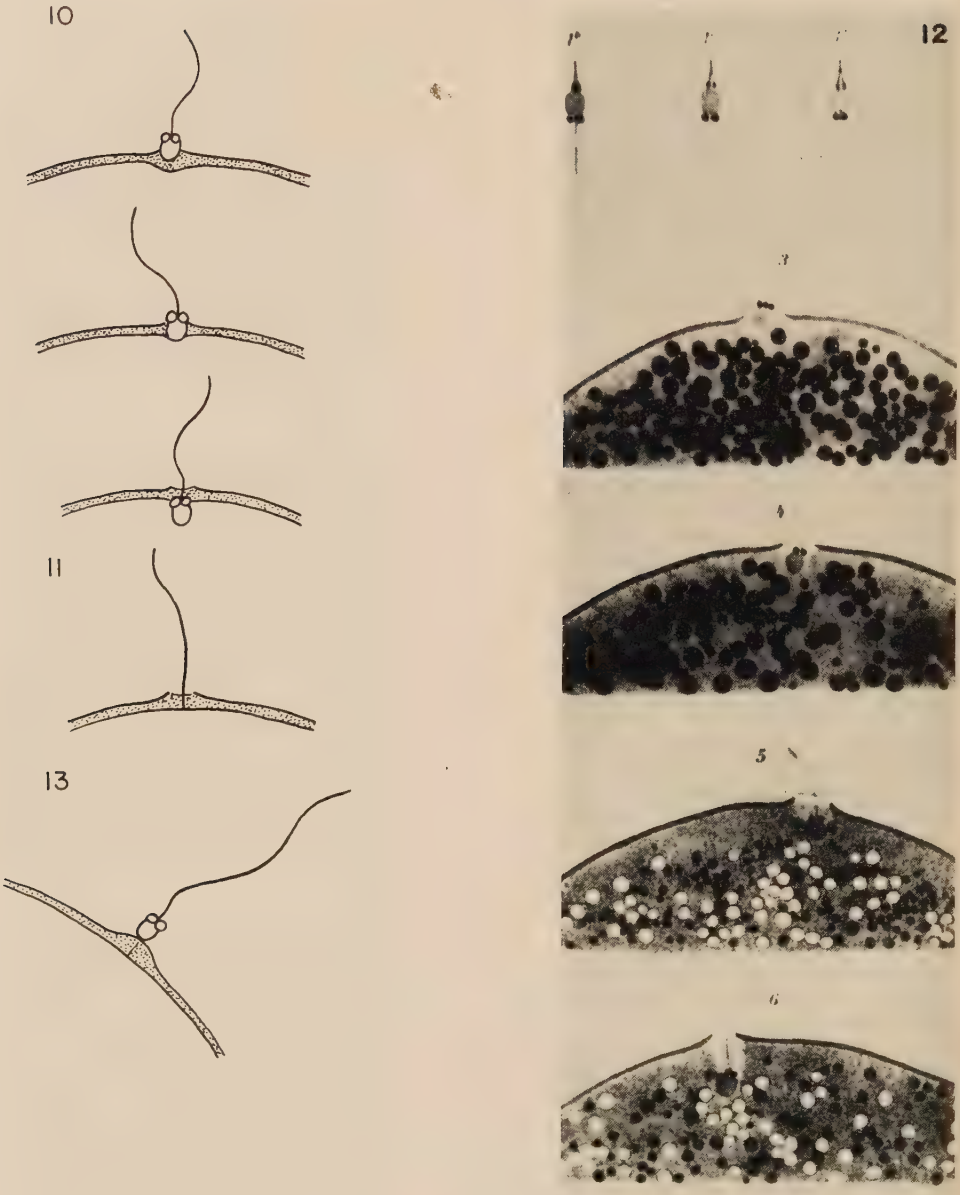

Fig. 10. Sketches of 3 stages in the progress of the *Mytilus* spermatozoan through the covering layers of the egg. Sketched from oil immersion phase contrast observation of living eggs.
Fig. 11. After engulfment of sperm head, opening about 3μ in diameter is left in vitelline membrane. Sketched from living egg (phase contrast oil immersion).

the vitelline membrane is judged by the fact that the polar bodies, which are normally flattened between the vitelline membrane and the egg surface (Fig. 8), round up and move away from it (Fig. 9) after short exposure to a strong lysin preparation. This is interpreted as indicating that the vitelline membrane possesses a major part of the tensile strength belonging to the surface coats.

In spite of the obvious desirability of observing the acrosome breakdown in normal fertilization, this has so far proved impossible, because of the intense activity of the spermatozoa. However, fixation immediately (2 sec.) after insemination shows that the spermatozoa penetrate the jelly layer very rapidly and the acrosomal reaction takes place on contact with the vitelline membrane. At the earliest moment after fertilization at which observation is possible in living material, the head of the fertilizing spermatozoan, minus its acrosome, is seen closely fixed and immobile against the membrane, while the tail may show small-scale, quivering movements. For $2\frac{1}{2}$ to 3 minutes (at 15° C), no visible progress is made, and then the sperm head begins to sink through the surface layers. In most cases it is possible to observe a slight bulging of the membrane at this time, together with a transitory widening of the hyaline layer (Fig. 10). Under the best optical conditions, with phase contrast oil immersion, an opening in the vitelline membrane can be seen after the sperm head has passed through it (Fig. 11); this is identical with the figure in Meves' 1915 paper [9] showing sections of *Mytilus* eggs immediately after fertilization (Fig. 12). In certain cases in which spermatozoa remain attached to the surface longer than normally, there is often a marked bulging of the vitelline membrane (Fig. 13), which is interpreted as an indication of local softening.

DISCUSSION

In his study on the lytic effects of the *Mytilus* sperm lysin, Berg [1, 2] does not offer any suggestions as to its location in the intact spermatozoan. Among the results obtained with other materials, however, there are several suggestive points which fit well with the conclusion that the egg-membrane lysin of mollusc spermatozoa is located in the acrosome. In particular, Tyler [15] found, as demonstrated by electron micrographs (p. 210) of *Megathura*

Fig. 12. Reproduction of part of plate from Meves (1915). Drawings 3 and 4, of *Mytilus edulis* eggs fixed $2\frac{1}{2}$ minutes after insemination; 5 and 6, fixed at $4\frac{1}{2}$ minutes. In drawing 5, note vitelline membrane separated from surface of hyaline layer.
Fig. 13. Sketch of supernumerary spermatozoan which remained attached to egg surface for about 10 minutes. Fertilization cone-like bulge is attributed to softening of the vitelline membrane by the sperm lysin.

spermatozoa which had been treated with sea water at pH 9, that the breakdown of the acrosome releases the lysin into solution.

Krauss [7], using the same material, also found the lysin to be released in progressively greater amounts from a dense sperm suspension kept at 1° C for 12 days, although the total amount released represented only about 20 per cent of the total lysin content of the suspension (as determined by extraction at the end of the experiment). This agrees with microscopic observations made on the spermatozoa of various species (among echinoderms, molluscs and annelids) which show considerable proportions of broken down acrosomes in aging and moribund sperm suspensions. It will also be recalled that acrosome breakdown was induced by exposure to low temperature in *Mytilus* spermatozoa. From the facts that some lysin is found in the supernatant from dense suspensions of fresh spermatozoa, and is released from spermatozoa under other "physiological" conditions, or at least without totally disrupting the cells, Krauss drew the conclusion that part of this substance is located at or near the surface of the spermatozoa.

Among Krauss' results with *Megathura* material there is one point which does not accord with the general scheme that egg-water induces the acrosome reaction, which releases lysin. Krauss found no difference between the lysin titers of supernatants from egg-water-treated and non-egg-water-treated sperm suspensions. The writers' experience with molluscan gametes has shown [3] that there is a wide range in the readiness with which the spermatozoa of different species undergo the acrosome reaction in response to the stimulus of egg-water. It has further been found that spawned gametes are decidedly more reactive than those obtained by extirpating and mincing the gonads. Finally, experiments with *Scutus scapha*, a Japanese member of the family *Fissurellidae*, to which *Megathura* belongs, as well as with other of the *Gastropoda*, indicate that the spermatozoa of this group are consistently unresponsive to the stimulus of egg-water. It is therefore suggested that microscopic examination of such *Megathura* sperm suspensions as those represented in Krauss' Table 10 would show approximately equal numbers of reacted spermatozoa in the presence and absence of egg-water.

The strong lytic activity shown in Berg's [2] Table 1, when concentrated sperm suspensions were brought into contact with the egg membranes, is precisely what would be expected if many reacting spermatozoa released their lysin at the membrane surface. The slight effect of the supernatants of such suspensions also agrees with that found in the experiments of Series (2), reported above.

With respect to the dissolving action of the *Mytilus* sperm lysin on a "ce-

menting substance'' which Berg describes as holding the blastomeres of the *Mytilus* egg together after cleavage, the following interpretation is offered. It is suggested that this substance makes up the ''hyaline layer'' which is present in the unfertilized egg, underlying the vitelline membrane. In fact, Berg's reported observation indicates that he was dealing with this substance: ''Fertilized eggs which have had the membranes removed, often become sticky and adhere to the bottom of the containers. Addition of a sperm extract causes the adhesiveness to disappear'' (p. 132). Regardless of the possible role of this layer in holding the blastomeres together after cleavage, it seems clear that it constitutes one of the barriers surrounding the unfertilized egg, which the spermatozoan must penetrate in order to reach the cytoplasm. If this interpretation is correct, Berg's second lytic activity (that of the ''cement lysin'') becomes of primary importance in the fertilization process.

With respect to the role of the lytic substance in fertilization, there seems to be no reason to question Tyler's [14] statement: ''The manifest action of the lysin on the membrane barriers in molluskan... eggs implies that it is instrumental in enabling the individual spermatozoön to penetrate to the surface of the egg proper'' (p. 210).

In *Mytilus*, at least, this statement can now be prefaced with another: The reaction of the spermatozoan acrosome on contact with the egg surface results in its breakdown, releasing one or more lytic substances.

SUMMARY

When *Mytilus edulis* spermatozoa are exposed to *Mytilus* egg-water, low temperature or an excess of Ca^{++}, their acrosomes break down and a slender filament is extruded. In order to determine whether this reaction releases an egg-membrane lysin, dense sperm suspensions were stimulated by excess Ca^{++}, and the reacted spermatozoa were centrifuged out of suspension and extracted with sea water after being frozen and ground in a mortar. Tests of this extract and of the supernatant containing the acrosome material, for their lytic power on the vitelline membranes of plasmolyzed *Mytilus* eggs, showed that most of the lysin was contained in the supernatant.

Conversely, the supernatant from unreacted sperm suspensions was compared in the same way with the extract of the intact spermatozoa. In this case the extract showed strong lytic capacity and the supernatant, practically none. These reciprocal experiments are interpreted as indicating that the acrosome contains an egg-membrane lysin which is released at the time of acrosome breakdown.

Phase contrast oil immersion observation of sperm entrance in *Mytilus* shows that an opening about 3μ in diameter is left in the vitelline membrane after the fertilizing spermatozoan has been engulfed by the egg cytoplasm. This vital observation coincides exactly with Meves' 1915 figures of the process in eggs fixed during sperm entrance.

The writers acknowledge with thanks the unfailing cooperation of the Staff of the Misaki Marine Biological Station. One of us (J. R. C.) wishes to express his most sincere appreciation to Prof. Katsuma Dan and the Tokyo Metropolitan University for the many kind favors extended during his stay in Japan. Gratitude is also expressed to Prof. I. Tomiyama and the Staff of the Misaki Marine Biological Station for their generous hospitality in making available to him the facilities of their station.

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THE FUNCTIONAL DIFFERENTIATION OF EMBRYONIC CHICK THYROID IN ROLLER TUBE CULTURES[1]

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Some recent work on the physiological differentiation of the thyroid in tissue culture includes that of Gaillard [2], Carpenter, Beattie and Chambers [1], and Gonzales [3].

Gaillard found that thyroids in tissue culture acquired the capacity selectively to absorb I-131. Carpenter *et al.* also studied the I-concentrating function as correlated with histological differentiation of the thyroid rudiment in tissue culture. Gonzales demonstrated, by the method of filter-paper radio-chromatography, the initiation of formation of monoiodotyrosine (MIT), diiodotyrosine (DIT) and thyroxine (Tx)[3] in explants of thyroid rudiments of the chick. He found that after a brief period of production of these iodinated amino acids the activity of the explants was greatly reduced.

A possible reason for this decline is the poor oxygen conditions which prevail in Maximow cultures. In this method, the tissue is embedded in a rather thick clot and the oxygen and nutriment transport is limited by the rates of diffusion of these substances.

It is well known that the roller tube method of cultivation, while not offering ideal conditions for differentiation, nevertheless does provide conditions for efficient nutrient-waste exchange [7]. For these reasons the roller tube method was applied to the study of the functional differentiation of the embryonic chick thyroid.

PROCEDURE

Seven- to 7½-day chick embryos (New Hampshire × White Leghorn) were removed from the eggs and collected in Petri dishes containing a mat of filter paper moistened with Tyrode. At this stage of development the thyroids have not yet acquired the capacity to synthesize Tx and DIT [5]. When all the embryos had been collected, the thyroid lobes were carefully dissected out and placed in a Petri dish containing

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[3] In collidine-water, the solvent system used throughout this study, thyroxine and triiodothyronine migrate in a single spot and are not separated from each other [4].

sterile Tyrode solution. Next, the entire lobes were transferred to the inner walls of the tubes, or in some cases to long, narrow coverslips which were later put into tubes. A thin plasma clot was formed to hold the explants in place. It was found that 5 explants were sufficient to yield a good chromatogram after a few days in culture. After the plasma had clotted well, 1 cc of nutrient fluid was added to each tube. The fluid was prepared according to the method of Waymouth and White [6], except that beef serum was used instead of horse serum because of its availability. After the cultures had been set up, they were put into the incubator (37° C) in a roller drum which turned the tubes 12 times per hour.

Twenty-four hours before the harvesting of the cultures for chemical analysis, 5 micro-curies of carrier-free I-131 was added to each tube by means of a micropipette. In this way possible radiation effects were canceled out since in every case the cultures were exposed to the same quantity of I-131 for the same length of time.

The medium and explants were analyzed separately according to the method previously described [3]. Essentially the analysis consisted of: (1) precipitation of the proteins, (2) hydrolysis of the washed precipitate, (3) extraction of the hydrolysate with butanol, (4) one-dimensional filter-paper chromatography of the extract and (5) exposure of X-ray film to the chromatogram for localization of radioactivity.

The strips of exposed film were run through a synchronous motor-driven linear densitometer, the output of which was connected to a recording potentiometer. A rough estimation of the amount of radioactive substance was obtained from the resulting curves.

In addition to the cultures that were set up for the chemical analyses, other cultures were made under similar conditions for fixing and staining in order to compare the functional state of the explants with their histological condition. For histological examination, whole mounts were made of cultures after they had been fixed and stained by the periodic acid Schiff (PAS) method.

RESULTS AND DISCUSSION

In general, the roller tube method proved far superior to the Maximow method in the maintenance of functional differentiation of the embryonic chick thyroid as judged by the production of Tx and other iodinated amino acids. Morphologically the rudiments differentiated as well in Maximow slides as they did in roller tubes. However, cultivation in the latter resulted in a more intense synthetic activity and also in a longer duration of the period of activity.

Histological observations.—As noted in the previous section, the cultures set aside for histological examination were prepared as whole mounts. This was made feasible by the fact that a few days after explantation, the explant, because of cell migration, begins to flatten and spread. Thinning out of the original explant follows such a course that by the end of 6 days of cultivation numerous localized collections of PAS-positive material, presumably colloid, are observable in the center of the culture (Figs. 1, 3, 5). At higher magni-

fications cells were observed surrounding the larger colloid accumulations in a follicular arrangement. The droplets of PAS-positive material ranged in size, in the 6-day cultures, from less than 2 micra to 12 micra in diameter. The vast majority were of a size intermediate between these two extremes.

After 14 days cultivation the cultures were considerably thinner and more spread out. In some cases, epithelial sheets had migrated out of the explants (Fig. 2). PAS-positive droplets were not observed in the migrating epithelium except a few in the more proximal parts of the sheets. The 14-day cultures exhibited a considerable increase in the size of the PAS-positive droplets (Figs. 4, 6) over those in the 6-day cultures (Figs. 3, 5). Diameters ranged from less than 2 micra to 40 micra with most of the droplets having an intermediate diameter. In the larger follicles of the 14-day cultures the colloid appeared to be forcing itself through the interstices between the follicular cells. This was probably due to the fact that so much of the stromal tissue, which would ordinarily support the follicle, had migrated away from the explant.

Results of the chemical analyses.—By the end of 3 days in culture, MIT, DIT and Tx were present in the explants (Fig. 7). Two other spots of radioactivity were consistently found in the chromatograms. One was the spot corresponding to iodide (I, Fig. 7). This iodide is not to be confused with the free iodide in the medium, since the chromatograms were made with hydrolysates of protein precipitates which had been washed. Thus, the iodide appearing in the chromatograms is probably that freed during hydrolysis. The remaining radioactive spot (X, Fig. 7) is a front-running component, the nature of which is not known.

From day 3 through day 6 there is a steady increase in synthetic activity in the explants (Fig. 7). This increased production of iodinated compounds really reflects an increase in *rate*, since all cultures were exposed to the I-131 for the same length of time, viz. 24 hours. Thus, the area under the curves depends on that quantity of radioiodine incorporated during 24 hours and not on the total quantity of the compound formed since the beginning of cultivation.

The chromatograms in Fig. 7 (and Fig. 8) are of explants and not of the medium. None of the iodinated amino acids was ever found either in the protein fraction or in the non-protein fraction of the medium. Thus, even though there is a significant amount of synthetic activity within the explant, the results of secretory activity are not observable by the methods used in this study.

The present data do not entirely exclude the possibility of a simple exchange

between radioiodine in the medium and non-radioactive I on preformed Tx. However, there are suggestions that no significant amount of exchange was taking place under the conditions of cultivation. In the two cases in which the cultures were contaminated during the first refeeding (after 4 days *in vitro*) no Tx or DIT could be found on subsequent analysis. This could be interpreted in several ways. Either the bacteria destroyed the Tx and DIT which was present at the end of 4 days and also the labeled amino acids as they were formed, in which case the question of exchange would be left open; or the bacteria interfered with the synthetic activity of the cells without destroying any significant amounts of Tx and DIT, in which case no detectable exchange takes place between I-131 in the medium and stable I on the amino acid molecule.

It is important to note (Fig. 7) that there is a peak of activity reached at 6 days. The 7-day explants were less active than those which had been in culture only 6 days. In order to find out whether the explants would become inactive after a longer period of cultivation some cultures were left in for 14 days. The chromatograms in Fig. 8 are from 6- and 14-day cultures. This shows that there was some loss of activity between day 6 and day 14. However, the explants were still producing MIT, DIT and Tx even after 14 days *in vitro*. Although there was a reduction of activity during this period, histologically the explants made progress as evidenced by the fact that the follicles were larger in the 14-day cultures (Fig. 8).

In a previous paper [3] we noted that Maximow cultures declined rapidly after initiation of function (production of Tx and DIT). Carpenter *et al.* [1] found that thyroid explants continue to concentrate I-131 even after 8 days in Maximow slides. The techniques of these workers differed in two important respects. The age of the donor embryos was different in the two studies, Carpenter *et al.* having used embryos one day older. Furthermore, Carpenter

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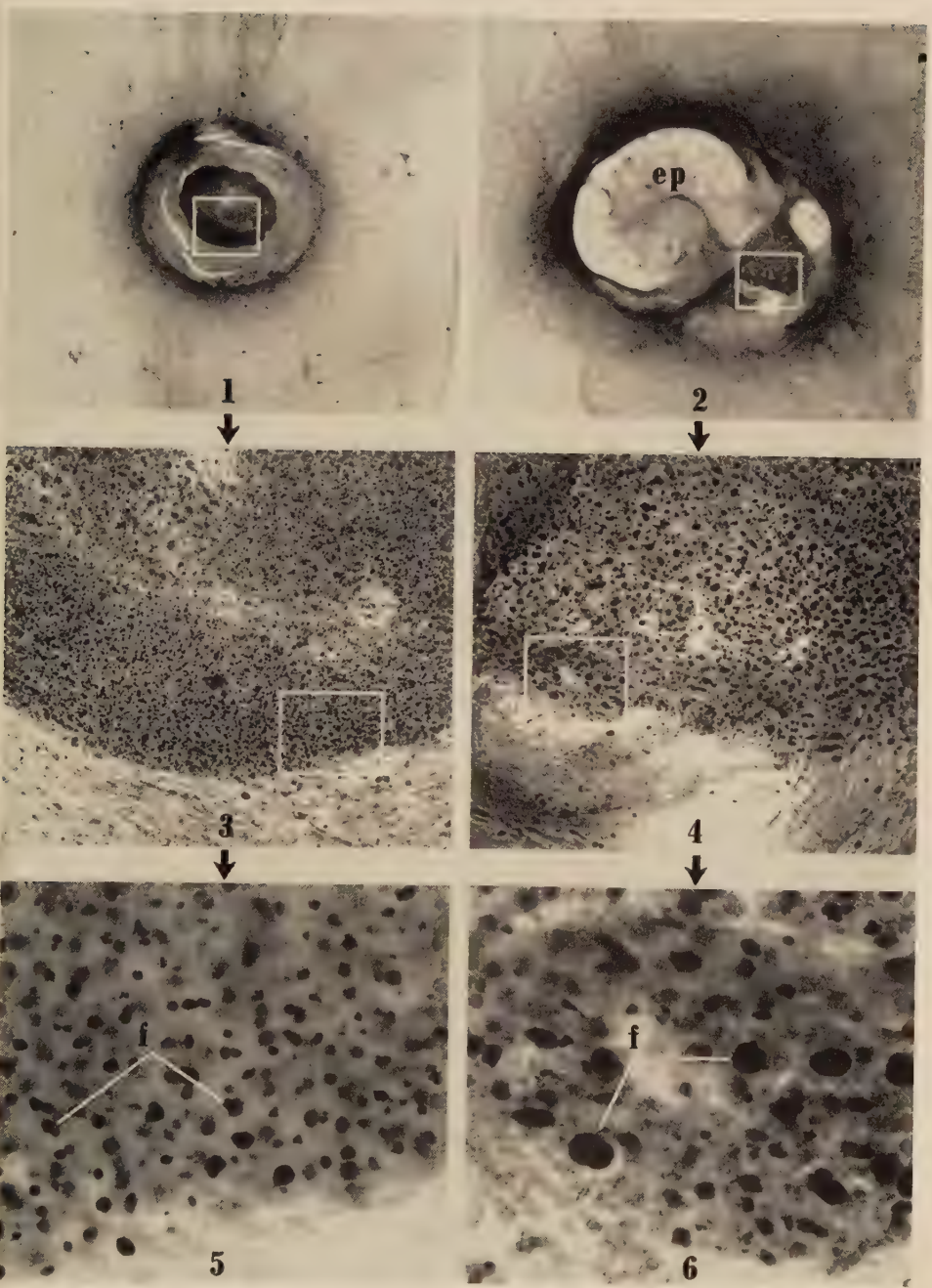
Figs. 1–6. Photomicrographs of 6- and 14-day cultures of embryonic chick thyroid explanted from 7½-day embryos. Cultures illustrated were prepared as whole mounts after periodic acid Schiff' staining. *ep*, epithelial outgrowth; *f*, follicles.
Fig. 1. Overall view of a 6-day culture. ×12.
Fig. 2. Overall view of a 14-day culture. ×12.
Fig. 3. A portion of the boundary between the original explant and the zone of outgrowth in a 6-day culture. At this magnification the distribution of the PAS-positive droplets is evident. This photomicrograph is of the area within the white rectangle in Fig. 1. ×85.
Fig. 4. A portion of the boundary between the original explant and the zone of outgrowth in a 14-day culture. Notice the increased size of the colloid-filled follicles. This is a photomicrograph of the area within the white rectangle in Fig. 2. ×85.
Fig. 5. Higher magnification of the area within white rectangle in Fig. 3 (6-day culture). ×375.
Fig. 6. Higher magnification of the area within white rectangle in Fig. 4 (14-day culture). ×375.


ep
1
2
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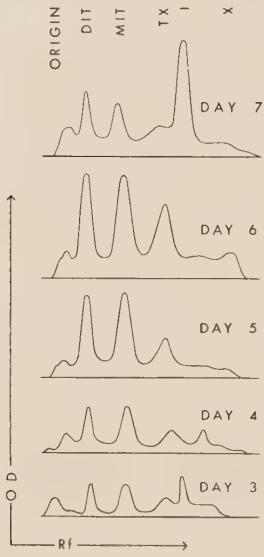
et al. explanted thyroid lobes on the surface of the medium, whereas Gonzales embedded them within the medium. These differences in technique in addition to the differences in indices of function of the explants may account for the lack of agreement in results. The fact that functional differentiation as



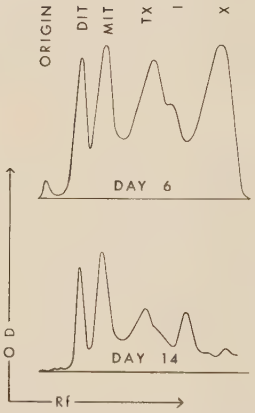


Fig. 7 (left). Chromatograms of 3-day through 7-day cultures. DIT, diiodotyrosine; MIT, monoiodotyrosine; Tx, thyroxine; I, iodide; X, unknown, front-running component.
Fig. 8 (right). Chromatograms of 6-day and 14-day cultures. Symbols as in Fig. 7.

judged by the production of Tx and DIT proceeded well in roller tubes, even though the explants were embedded in a clot, suggests that the agitation of the medium and/or alternate exposure of the clot surface to the gas phase and to the liquid phase are of some importance for the function of the explants.

SUMMARY

1. Whole thyroid lobes from 7–7½-day chick embryos were explanted to roller tubes for a morphological and physiological study of differentiation *in vitro*.

2. Filter-paper chromatography and autoradiography of the chromatograms, after introduction of radioiodine into the culture medium, showed that monoiodotyrosine, diiodotyrosine, thyroxine, iodide and an unknown, radioiodine-labeled compound were present after 3 days *in vitro*.

3. The rate of production of the iodinated amino acids increased with ime *in vitro* and reached a peak about day 6. At day 14 the explants were still producing these compounds, but at a lower rate.

4. The iodinated compounds were found only in the cultures and were not detectable in the medium.

5. By day 6 numerous PAS-positive droplets, presumably colloid, were found evenly distributed throughout the central part of the cultures. At day 14 the colloid accumulations were larger.

6. Epithelial outgrowths showed colloid formation only in the more proximal parts of the migrating sheets.

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INFLUENCE OF HUMAN CORD EXTRACTS ON CHICK EMBRYONIC FIBROBLASTS GROWN *IN VITRO*

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Although a stimulating action of the placental serum or even the placenta extracts has often been reported in tissue culture, no such statement has been made, to our knowledge, about the cord extract itself. The present report gives some results on the properties of these extracts.

TECHNIQUES

Umbilical cords freshly obtained are cut into fragments about 2 cm long and extensively washed in Ringer's saline solution until complete removal of the blood. The fragments are drained and collected in an electric grinder in the presence of as many ml of Tyrode solution as grams of tissue. After grinding, the pulp is centrifuged at 6000 rpm for 20 minutes. The supernatant, very clear, is our 1 to 1 concentrated solution of cord extract.

Such extracts have been tested at the respective dilutions of 1/2, 1/4, 1/5, 1/10, 1/20 in comparison to identical solutions of embryonic extract, on hanging drop cultures of chicken fibroblasts kept 4 days on a thin film of plasma, without change of nutrient. Controls were established by growing fibroblasts in the same manner but the extracts were replaced by an equal volume of Tyrode solution. The average increase in area for each series of cultures was estimated every day by means of an ocular micrometer and expressed in function of time. Mass cultures on glass in roller tubes where the medium was changed every three days [4], gave us information as to the nutritive qualities of the extracts.

RESULTS

The effect of umbilical cord extract at whatever concentration it is used (from 1/2 to 1/20) is characterized, in hanging drop cultures by an intense cell migration. From the beginning, the cells are spreading widely in the medium covering an area which increase is proportional to time. After 4 days of continuous cultivation, the average surface covered by cells growing in

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the cord extract is about 1.5 times larger than the surface obtained with the embryo extract and 4 times larger than in saline solution. Even though the medium is not renewed the cultures appear in excellent morphologic condition and some mitoses can still be seen. Comparatively, degenerative areas are observed in cultures made with embryo extract which would indicate that cord extracts provide a good maintenance medium. These facts

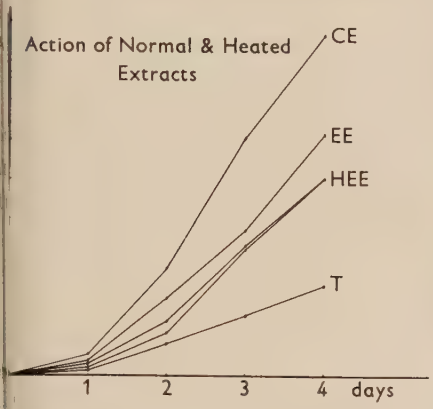


Fig. 1 (left). *CE:* cord extract, *EE:* embryo extract, *HEE:* heated embryo extract, *T:* Tyrode solution. Both cord and embryo heated extracts give an identical cell migration after 4 days of cultivation. The value expressed on the chart is still very significant compared to the controls.

Fig. 2 (right). Roller tube culture after 2 weeks, maintenance in a 1/10 cord extract medium.

have been established through a half dozen experiments which gave very constant results.

We tried to eliminate the stimulating factor from our extracts by a 15-minute exposure in a boiling water bath. Whereas an abundant protein precipitate appears in the embryo extract, the cord extract remains practically clear. After centrifugation, such extracts still enhance the migration in fresh cultures; after 96 hours the average area is still twice as large as in the controls in saline (Fig. 1).

Long term cultures, in roller tubes, showed that this stimulating action of both fresh and heated extracts is only temporary. At the best the cultures could survive 3 weeks and then rapidly degenerated (Figs. 2, 3). Therefore the factor involved is not nutritional. What then is its exact nature?

In order to get information on the molecular size, a 1 to 10 dilution of cord extract was dialyzed for 10 days in a cellophane bag against a glucose-

TABLE I

Action of dialyzed extracts.

	24 h	48 h	72 h
Cord extract 1/20	1.8	9	19
Dialized c. e. 1/20	0.8	6.5	13.6

The numbers express the relative increase in area for an average of 60 cultures. Most of the activating factors are retained.

TABLE II

Effect of hyaluronidase on the stimulating action of decreasing concentrations of hyaluronic acid.

Hyaluronic acid (H.A) mg/ml	H.A. alone	H.A. hyaluronidase (90 T.R.U.)
6	20	19
3	14	13.8
0.5	8	8

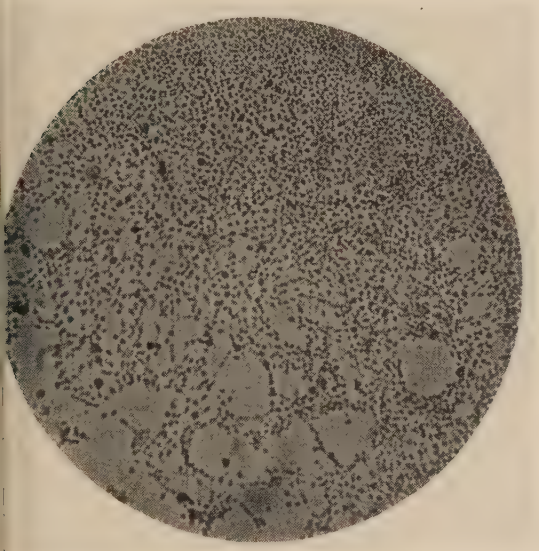
Each figure expresses the relative increase in area for an average of 10 cultures after 3 days of cultivation. While the migrating area becomes smaller with decreasing amounts of hyaluronic acid, one cannot note any specific intervention of hyaluronidase when this enzyme is introduced in the medium.

Ringer solution (1 g of glucose per 1000) according to a procedure developed by Fischer [2]. The resulting product tested in hanging drop cultures in comparison to undialysed extracts showed that a great part of the accelerating effect was retained (Table I). The active compound is therefore of a high molecular weight.

The rich composition of the umbilical cord in hyaluronic acid suggested a possible action of this larger molecular product. A solution which is equivalent to the hyaluronic acid content of the normal human cord (about 6 to 8 per cent of the dry weight) was made at the concentration of 6 mg of H.A. per ml of Tyrode solution. The fragments of tissue placed in this medium responded practically as well as with the cord extract itself (Table II). After 3 days the average surface of the cultures was only slightly better with the total cord extract. If we consider that a part of the hyaluronic acid is lost to the cells by combining with the proteins of the medium, the results obtained are particularly outstanding. This combination could be observed in

the plasma clot under the form of a slight cloudiness caused by fibrillar precipitations. Such precipitations did not occur with the cord extracts even in their highest concentration.

Since hyaluronic acid appeared to be the stimulating factor it was interesting to test comparatively the action of hyaluronidase. We used a carefully controlled testicular hyaluronidase titrating 90 turbidity units per ml. This



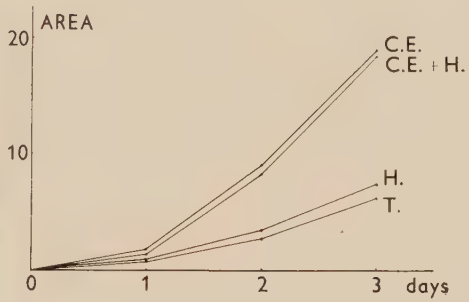


Fig. 3 (left). Roller tube culture after 3 weeks' maintenance in the same medium. Fig. 4 (right). Action of hyaluronidase on cord extracts. *CE:* cord extract, *H:* hyaluronidase, *T:* Tyrode solution. The addition of hyaluronidase does not affect the action of the cord extract.

solution was mixed in equal parts with a cord extract diluted 1/10 and with a hyaluronic acid solution (6 mg/ml) corresponding to the 1/10 dilution of cord extract. The mixtures were made 15 to 30 minutes prior to cultivation. In such conditions the hyaluronic acid and similar components were totally depolymerized during the latent period of the cultures. We also tried the same concentration of hyaluronidase on decreasing concentrations of hyaluronic acid.

The test performed on hanging drop cultures showed no neutralizing effect of the hyaluronidase either on the cord extracts (Fig. 4) or on the hyaluronic acid solutions (Table II). After 3 days the average areas were practically the same for the cultures made with or without the enzyme. The findings may be related to an observation of Kulonen [3] who found that hyaluronidase had no effect on the stimulating action exerted by hyaluronic acid on the growth of tadpoles.

A few proteins are precipitated in the cord extract when an equal volume

of 1 per cent acetic acid is added. These proteins washed 3 times in 1 per cent acetic acid, then washed again in Tyrode solution have been tested in hanging drop cultures. No definite increase of the surface could be recorded after 3 days, although a slight activation could be noticed in the first 48 hours. Nevertheless, the fact that heat does not fundamentally affect the properties of the cord extract shows that such proteins have only a small significance.

DISCUSSION AND CONCLUSIONS

To conclude, hyaluronic acid appears as the main factor involved in the accelerating effect of the cord extract *in vitro*. Very likely it is rather a spreading property than a growth promoting action which is observed in tissue culture. Supporting that view is the fact that migrating cells on slides are always less compact in cord extract and that long-term cultures cannot be extended over 3 weeks. Furthermore, this stimulating action can be attributed to the elementary molecule of hyaluronic acid since it is not affected even after depolymerization by hyaluronidase.

Besides hyaluronic acid, the small molecular compounds and acid acetic protein precipitates have not much significance. Among the other components which are known to enter the composition of the umbilical cord are the chondroitinsulfuric acid and two polypeptides; the polypeptides may be slightly active, but as for the chondroitinsulfuric acid, Balasz and Holmgren [1] have shown that sulfonated mucopolysaccharides are rather toxic and suppress the growth of embryonic tissue *in vitro*.

Our results may present some interest in relation with other findings in cancer research. It is well known, for instance, that serum from cancerous patients has a high content in mucoproteins [8]. Pirie [5] reported in 1942 the presence of a mucin-like substance in the Rous sarcoma in which Warren *et al.* [7] have later traced hyaluronic acid. More recently, Thierry [6] also reported a high content of hyaluronic acid in the infectious myxome of the rabbit (*Sanarelli* myxome). This compound can probably be found in other neoplasms. A systematic research of the effect produced in tissue culture by mucopolysaccharides extracted from different tumors may prove to be of high interest.

SUMMARY

The effect of human umbilical cord extracts on hanging drop cultures of chicken fibroblasts is characterized by an intense cell migration. The stimulating factor has been identified as being the hyaluronic acid. Even after depoly-

merization by hyaluronidase the stimulating action is retained. This would indicate that only the elementary molecule of hyaluronic acid is involved. No nutritional effect could be demonstrated on long-term cultures and very likely it is rather a spreading property than a growth promoting action which is observed.

We wish to express our thanks to Dr. P. Boquet who supplied us with hyaluronidase and a highly purified sample of hyaluronic acid (Wyeth Institute of Biochemistry). Also, our gratitude is due to Dr. H. B. Fell, F.R.S. and Dr. M. Webb, from the Strangeways Laboratory, Cambridge, England, and to Dr. M. R. Murray from Columbia University, New York, for their invaluable advice and criticism.

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QUANTITATIVE OBSERVATIONS ON MITOCHONDRIA FROM SECTIONS OF GUINEA-PIG MAMMARY GLAND

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Before the methods for isolating mitochondria by centrifugation and by examination of sections under the electron microscope were introduced, there was little reliable information of a quantitative kind on mitochondria in the literature, especially with reference to cells in different functional states. Thin filaments, rods or granules scattered in large numbers in the cytoplasm are not easy to measure in stained preparations under the light microscope. Moreover, the fact that mitochondria were known to be labile structures, motile in the living cell and easily distorted, must have discouraged many cytologists from attempting detailed measurement. Recently a new approach to the problem has been introduced by Allard, Mathieu, de Lamirande and Cantero [4] and Shelton, Schneider and Striebich [19] who have estimated the number of mitochondria per cell or per gramme of fresh tissue, homogenized and centrifuged in the unfixed state. The results with liver are particularly encouraging and this method may prove to be suitable for other tissues and organs. At least it has the merit that measurement can be made from samples of homogenized tissue used at the same time for biochemical studies. However, where an organ or tissue contains numerous cell types each varying in function and in activity, it seems essential that information from the biochemical approach should be supplemented if possible by measurements derived from histological material. Both methods are bound to introduce errors but the new impetus given to the study of the structure of mitochondria by the electron microscope leads one to suppose that quantitative information from the latter source is worth pursuing, even if it provides no more than confirmation of the measurements derived from homogenization of the living tissue.

A convenient tissue with which to test whether fluctuations of metabolism are accompanied by significant quantitative changes in mitochondria is the mammary gland where the quiescent alveolar cells immediately before birth

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are called upon in the space of a few days to enter the phase of full lactation. According to Folley and French [9, 10] the oxygen uptake ($-Q_{O_2}$) of mammary gland slices is low in the rat at the end of pregnancy and the R.Q. (in glucose) is well below unity. The $-Q_{O_2}$ rises considerably by the first day of lactation to a maximum value of approximately 10 at mid-lactation. In homogenates and mitochondrial fractions of guinea-pig mammary gland a seven- to ninefold increase of succinic dehydrogenase and cytochrome oxidase activity has been recorded by Moore and Nelson [16]. It would clearly be of interest to know whether this striking example of accelerated cell activity is accompanied by any detectable changes in the mitochondria. The following observations are concerned with information obtained from sections of mammary tissues examined by the light miroscope and from electron micrographs.

MATERIAL AND METHODS

Guinea-pig material was chosen in preference to rat material because it can be cut more easily in paraffin, and because the non-lactating gland of the guinea-pig offers better opportunity for selecting blocks of glandular tissue uncontaminated with adipose tissue. This is important in relation to osmium tetroxide fixation. It was decided to make a preliminary investigation from stained paraffin sections. Schridde's fixation (see Lee, [15]) was used because by preserving fat droplets as well as mitochondria this method appears to cause less distortion of the mammary cell than would be the case with most other fixatives for mitochondria. The lactating mammary gland presents special problems in fixation owing to the large amount of fat present and the collapsible nature of the parenchyma. For large blocks to be preserved successfully it is essential to fix by arterial perfusion. The dichromate-formalin component of Schridde's method was perfused through the abdominal aorta at a point just above the origin of the iliac arteries, using intra-peritoneal Nembutal anaesthesia (30 50 mg/kg body weight). Paraffin sections cut at 2μ were stained with Heidenhain's iron haematoxylin using the partially oxydised aqueous solution recommended by Baker and Jordan [5]. It was found much easier to cut serial sections at 2μ with a rotary microtome if a bath of 25 per cent alcohol was set up in a perspex container, and sealed to the surface of the knife with modelling wax so that the fluid level just coincided with the cutting edge. This device borrowed from contemporary techniques for ultra-thin sectioning can be strongly recommended for producing paraffin sections of even thickness with minimum compression.

Fields selected at random were photographed at a magnification of $\times$ 1900 and the prints enlarged to give a final magnification of $\times$ 6000. In all cases it was necessary to check over the contours of the mitochondria, the cell walls and the nuclear membranes: at the same time care was taken to identify fat droplets as distinct from mitochondria. Using a Stanley, Allbrit precision planimeter the total area of each sectioned cell and the area of each nucleus were measured, and the area of cytoplasm calculated by subtraction. A count was made of the number of sections of mitochondria appearing in each cell, but no attempt was made to measure the dimensions of the

mitochondria since their boundaries at this magnification were not sufficiently precise.

The material used for electron microscopy was obtained from separate animals not included in the preliminary survey. Small fragments removed under Nembutal anaesthesia, after fixation in 1 per cent osmium tetroxide buffered to pH 7.4 according to Palade [17], were dehydrated and embedded in a 4:1 mixture of *n*-butyl:methyl methacrylate. This material was cut at 0.05μ on a rocking microtome with a glass knife and photographed at a magnification of $\times$ 2500 with a Philips electron microscope. The prints were enlarged to a final magnification of $\times$ 20,000. Table I*a* summarises the relevant data concerning the individual animals. It should be noted that the number of days of lactation following parturition was recorded accurately in each case but the age of the non-lactating material lay somewhere within an interval of 1–4 days preceding parturition.

TABLE I*a*

Guinea pig No.	Body weight kg	Condition	Days allowed for suckling	Histological method
1	0.92	lactating	2	Schridde
3	1.00	,,	3	,,
6	0.66	,,	3	,,
7	0.51	,,	$4\frac{1}{2}$	,,
8	0.87	,,	$4\frac{1}{2}$	,,
11	0.58	,,	2	buffered OsO_4
12	1.05	,,	1	,,
2	1.20	non-lactating	—	Schridde
4	1.40	,,	—	,,
5	0.86	,,	—	,,
10	1.02	,,	—	buffered OsO_4
13	1.20	,,	—	,,

RESULTS

The measurements which can be made from random sections of fixed material are of course two-dimensional. In view of the irregular shapes of the mammary alveolar cells and the elongated, twisted form of many of the mitochondria, there would seem to be little justification for attempting to calculate total volumes of the various cell components from their areas in sections. The question arises, therefore, as to whether ratios based upon a limited number of area measurements can be expected to give even an approximate indication of differences between the mitochondrial content of cells in pre-secretory and secretory states.

Three plaster models of cells conforming roughly in dimensions to enlarged photomicrographs of lactating cells were constructed. Models of the ellipsoidal nuclei were first cast in coloured plaster from a mould, and several hundreds of "mitochondria" were made by pouring plaster of another colour into short lengths of rubber tubing. The diameter of the "mitochondria" was kept constant but the lengths were made to vary in order to imitate the short rods and elongated filaments of the actual cells. Each model cell was built by pouring plaster at intervals into a mould, orientating the mitochondria at random and placing the nucleus roughly in the middle of the cell. In each case the volume of the nucleus and of the total mass of mitochondria was measured by displacement in water before including them in the mould. The total volume of the cell was also measured and from this the volume of cytoplasm consisting of uncoloured plaster was calculated. When plaster is soaked in water after setting it can be cut very easily with a hack-saw and there is sufficient cohesion between the various coloured components in the mass to give sharp contours in section. Moreover, the halves of the sectioned block can be re-united by a thin layer of fresh plaster and further sections made in different planes. Each model cell was cut in six planes at random. Tracings were made of one cut surface in each plane for the contours of the cytoplasm, nucleus and mitochondria, the areas of which were then measured with the planimeter.

The data in Table I concern two model cells of different shape containing closely similar amounts of mitochondria and a third cell in which there was a greater amount of mitochondria. It will be seen that a fairly reliable comparison can be made between such cells if the ratios of the area of mitochondrial substance to area of cytoplasm in random sections is used in place of the ratios of the actual volumes. Thus the values of B'/A' for the three cells are related according to the proportions 1 : 1.06 : 1.57, which compares favourably with the proportions 1 : 1.10 : 1.39 for B/A (volume of mitochondria per unit volume of cytoplasm) taking the cells in the same order. A fairly close comparison can also be made between the values for C'/A' and C/A, allowing for the fact that some mitochondria may be cut twice owing to their curvature. As might be expected, the nucleo-cytoplasmic ratio calculated from the area measurements is much less reliable as a guide to the true differences between the three models because the nuclear material is concentrated in one location in the cell whereas the mitochondrial substance is dispersed throughout the cytoplasm.

Before we can be reasonably certain that area measurements from electron micrographs are likely to distinguish variations in mitochondrial content

TABLE I

Comparative estimation of the volumes and number of "mitochondria" in plaster models of cells obtained from area measurements from 6 sections through each cell at random.

Model	Actual quantities					Measured estimates				
	Total vol. of cytoplasm, ml A	Total vol. of mitochondria ml B	Total no. of mitochondria C	B/A	C/A	Av. area of cytoplasm cm^2 A'	Av. area of sectioned mitochondria cm^2 B'	Av. no. of sections of mitochondria C'	B'/A'	C'/A'
1	961	109	152	0.113	0.158	88.29	9.19	23.6	0.104	0.267
2	966	120	160	0.124	0.166	91.97	10.16	28.3	0.110	0.308
3	1193	187	250	0.157	0.209	103.76	16.96	42.0	0.163	0.405
B/A	1:2:3 = 1:1.097:1.389									
B'/A'	1:2:3 = 1:1.058:1.567									
C/A	1:2:3 = 1:1.051:1.323									
C'/A'	1:2:3 = 1:1.153:1.517									

with the same degree of accuracy as shown in the models, it is necessary to examine the state of dispersion of the mitochondria in the models and in the actual mammary cells. This can be done by superimposing grids containing squares on the photographs or tracings, adjusting the sizes of the squares to conform with the magnifications of the photographs and models. The midpoints of the mitochondrial sections were taken, and frequency counts made of the number of grid squares containing no mitochondria, the number containing one mitochondrion, and so on up to a maximum of four mitochondria per square, which was a rare event in this material. Table II gives the results of a limited number of counts made on photomicrographs of stained sections and electron micrographs from lactating epithelium, together with counts on the tracings of model 1. Testing for independence in this contingency table, the total value of χ^2 is 8.69 for d.f. = 8. The counts are therefore not inconsistent with the proposition that the model is an approximate imitation of the lactating cells so far as the distribution of mitochondria is concerned. The grid counts show moreover that the distributions of the mitochondria in the fixed material as well as in the model are not at random. This is presumably due to interaction caused by the elongated shape of the mitochondria and possibly to other factors in the actual cells such as clumping under the influence of surface tension forces or streaming movements in the

TABLE II

Showing the frequency of the numbers of mitochondrial sections occupying the squares of grids superimposed on photographs or tracings as a test of similarity in distribution.

No. of mitochondrial sections per square	0	1	2	3	4
Model 1 (6 sections). . . .	133	112	52	8	1
Photomicrographs of lactating cells.	285	263	117	31	3
Electron micrographs of lactating cells	221	181	60	14	2

cytoplasm. The counts fit neither a Poisson nor a binomial distribution, and calculation of the index of dispersion (see David, [7]) shows that the mitochondria are more evenly distributed throughout the cytoplasm than would be the case if they were placed at random.

In the models an estimate of the total number of mitochondria present and of their total volume can be obtained by making use of the known total volume of cytoplasm, the average length and diameter of the mitochondria, and the average areas of mitochondrial substance and cytoplasm in the sections. Let A' = the average area of cytoplasm containing B' square units of mitochondrial substance: let a = area of a transversely sectioned mitochondrion of average diameter, and l = the average length of the mitochondria. If V = the total volume of cytoplasm, then the number of mitochondria in the whole cell $= (V \times B')/(A' \times a \times l)$ and their volume $= (V \times B')/A'$. Applying these two formulae to the data in Table I,

Model	C	B	Estimate of C	Estimate of B
1	152	109	139	100.0
2	160	120	142	106.7
3	250	187	260	195.0

we find that the error in the estimates is not more than about 11 per cent, and the relative differences between the models is confirmed.

Photomicrographs of Stained Sections

The information obtained from this approach (see Figs. 1 and 2) was confined to measurement in a series of lactating and non-lactating glands

TABLE III

Comparison of mammary alveolar cell size and content of mitochondria estimated from lactating and non-lactating guinea-pigs from photomicrographs of stained 2μ paraffin sections. Mag. × 6000.

	No. of animals	No. of fields examined	Total no. of cells measured	Average sectional area of cell, cm²	Average sectional area of cytoplasm cm²	Area of cytoplasm/area of nuclei	Average no. of sectioned mitochondria per cell section	Average no. of sectioned mitochondria per sq. cm. cytoplasm
Lactating series	5	75	531	33.24 ±12.21	26.13 ±10.03	3.67	31.08 ±11.23	1.21 ±0.24
Non-lactating series	3	28	277	17.47 ± 4.13	10.12 ± 2.75	1.38	12.21 ± 2.39	1.26 ±0.34

of (1) the average areas of sections of mammary alveolar cells and of their cytoplasm, (2) the average number of sections of mitochondria per cell appearing in these sections, and (3) the average number of sections of mitochondria per unit area of cytoplasm. Since the measurements relating to mitochondria are likely to be less accurate by this approach than those provided by the electron micrographs, it is not proposed to publish them in the form of a complete table. In fact, when it became obvious that any difference between the non-lactating and lactating cells with respect to the amount of mitochondrial substance per unit area of cytoplasm was likely to be small, the measurement of the non-lactating material was cut short in favour of detailed examination of the electron micrographs. A summary of the results from the stained paraffin material is given in Table III. It will be noted that the alveolar cell sectioned at random increases about two-fold in area as the cell changes from the non-lactating to the lactating state, and there is an increase by a factor of about 2.5 in the average area of the cytoplasm. Using the arc sine transformation of the values in the last two columns of Table III, it can be shown that there is a highly significant difference ($F = 99.12$, $n = 1$, $n_1 = 101$) between the two functional states of the cell with respect to the average number of sectioned mitochondria appearing in each cell, whereas the average number per unit area of cytoplasm remains about the same.

Electron Micrographs

At a magnification of × 20,000 the mitochondria of the mammary cells can be identified without difficulty (see Figs. 3 and 4) by their size and internal structure except in possible cases where a mere shaving of the periphery

of a mitochondrion might be indistinguishable from parts of the endoplasmic reticulum. There are no detectable protein masses in the cytoplasm comparable with secretory granules, and the fat droplets are usually quite distinct. For technical reasons, it is difficult to arrange for the complete boundaries of individual cells to be included in an electron micrograph. We have, therefore, measured this part of our material without reference to individual cells, taking cytoplasmic and nuclear areas purely at random but excluding, of course, the stroma and the myoepithelial cells where these could be identified with precision.

In order to measure the amount of mitochondrial substance within a particular area of cytoplasm, tracings were made of the mitochondria so that their outlines were placed end to end in a continuous chain. Thus the total area of mitochondrial substance was easily measured with the planimeter without the necessity for summing a whole series of individual small areas. In addition, the long and short axes of each sectioned mitochondrion were measured to the nearest half millimetre. Using the formula $\pi\ a \cdot b$ for the area of an ellipse where a and b are the semi-axes, it was possible to show that, although the contours of the sectioned mitochondria were usually somewhat asymmetrical and sometimes even quite irregular, the calculated estimate of the total area of mitochondrial substance differed by less than 5 per cent from the planimeter measurement. It was assumed, therefore, that the formula $2\pi\sqrt{(a^2+b^2)/2}$, which gives an approximate value for the length of the perimeter of an ellipse, might be used to give some idea of the total perimeter of the mitochondrial sections and so provide a two-dimensional measure of the interface between the mitochondria and cytoplasm. Unfortunately there is no mechanical device, other than the rather unsuitable instruments used for measuring distances on maps, by which the length of the mitochondrial interface might be measured more accurately.

In Tables IV and V three ratios have been calculated from the electron-micrograph measurements based upon unit area of cytoplasm rather than on area of cytoplasm per cell. These are B/A, the total area of mitochondrial substance appearing in 1 cm^2 of the electron micrographs at a magnification of $\times$ 20,000, C/A which represents the number of sections of mitochondria for the same unit area of cytoplasm, and D/A, the total perimeter of the sectioned mitochondria per unit area of cytoplasm. If the variances of these ratios are stabilised by an angular transformation as in the previous calculations for the stained material, it can be shown that the ratios C/A ($F=42.26$, $n=1$, $n_1=46$) and D/A ($F=35.79$, $n=1$, $n_1=46$) are significantly greater in the non-lactating series, whereas the ratio B/A does not differ significantly

TABLE IV

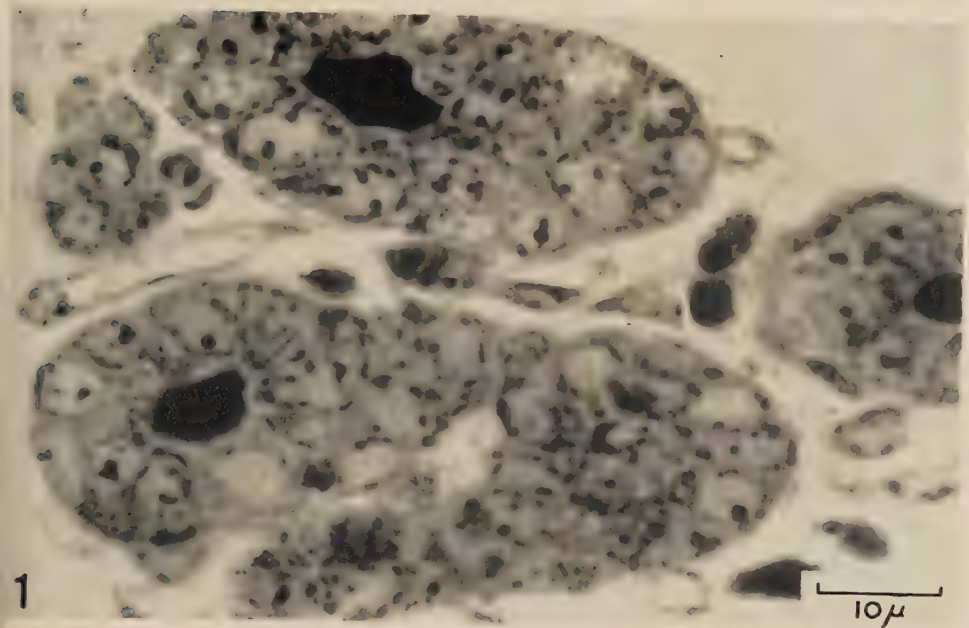
Measurements from electron micrographs at mag. × 20,000 of lactating alveolar cells.

Total area of cytoplasm cm^2	Total area of sections of mitochondria cm^2	Number of sections of mitochondria	Total perimeter of sect. of mitochondria cm	B/A	C/A	D/A
A	B	C	D			
449.80	31.38	43	149.73	0.07	0.10	0.33
346.19	14.35	27	74.03	0.04	0.08	0.21
427.08	18.83	22	76.89	0.04	0.05	0.18
309.14	14.63	27	73.50	0.05	0.09	0.23
407.22	23.25	31	114.49	0.06	0.08	0.28
398.61	22.28	31	100.09	0.06	0.08	0.25
395.10	21.25	40	107.08	0.05	0.10	0.27
411.16	21.77	37	123.06	0.05	0.09	0.30
478.31	23.51	33	110.76	0.05	0.07	0.23
395.54	16.16	31	96.55	0.04	0.08	0.24
484.99	28.15	47	172.05	0.05	0.07	0.35
457.37	14.48	33	86.62	0.03	0.08	0.19
558.75	21.05	43	136.13	0.04	0.07	0.24
403.99	16.03	29	95.56	0.04	0.07	0.24
495.25	22.80	38	138.49	0.05	0.08	0.28
523.69	20.82	66	150.75	0.04	0.13	0.29
442.96	23.28	51	142.30	0.05	0.12	0.32
560.42	32.11	51	163.64	0.06	0.09	0.29
463.67	29.48	50	176.45	0.06	0.11	0.38
490.60	23.95	47	142.22	0.05	0.10	0.29
483.91	31.10	80	195.64	0.06	0.16	0.40
485.05	22.00	53	138.06	0.05	0.11	0.28
481.13	18.20	52	123.65	0.04	0.11	0.26
524.91	22.08	42	130.65	0.04	0.08	0.25
			Mean	0.05	0.09	0.27
			$s_{\bar{x}}$	± 0.002	± 0.004	± 0.010

in the two cases. The results suggest, therefore, that whilst the amount of mitochondrial substance per unit area of cytoplasm remains about the same, there are more small mitochondria per unit area of cytoplasm in the non-

Fig. 1. Alveoli from non-lactating guinea-pig mammary gland showing mitochondria stained with Heidenhain's iron haematoxylin after Schridde's fixation.

Fig. 2. Portion of an alveolus from guinea-pig mammary gland on the third day of lactation showing mitochondria stained with Heidenhain's iron haematoxylin and fat droplets after Schridde's fixation.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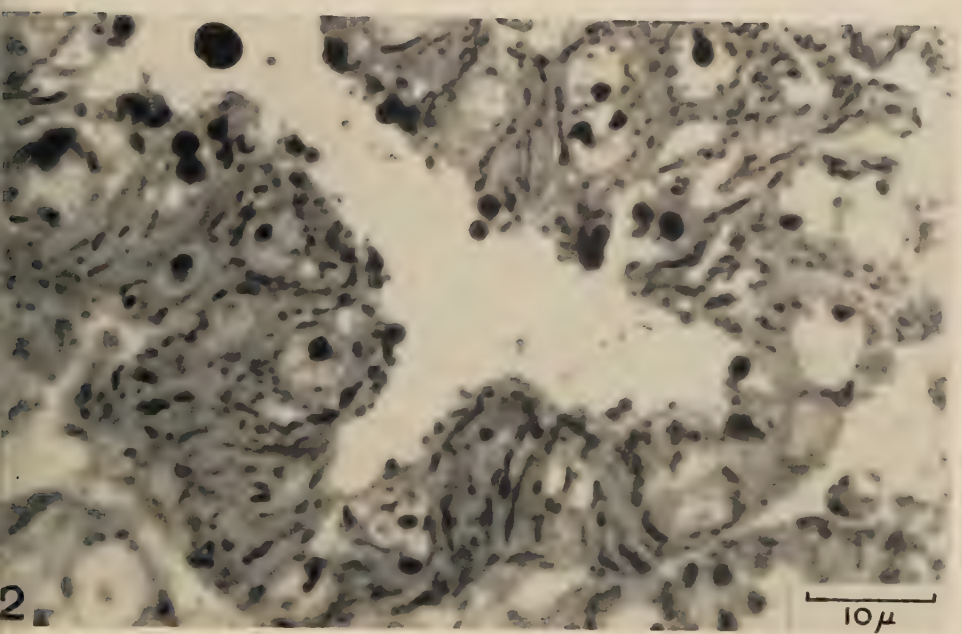
10μ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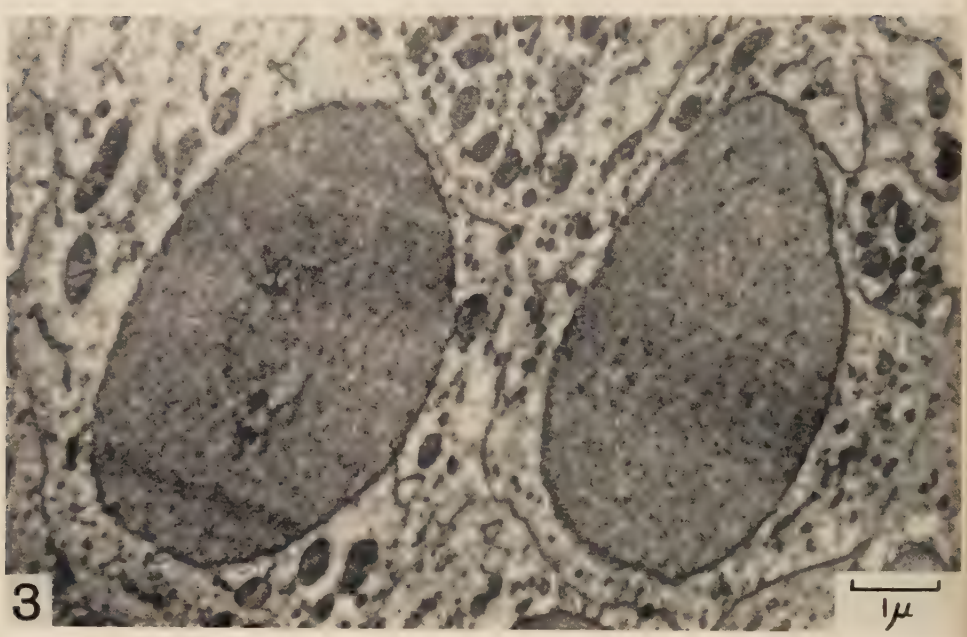
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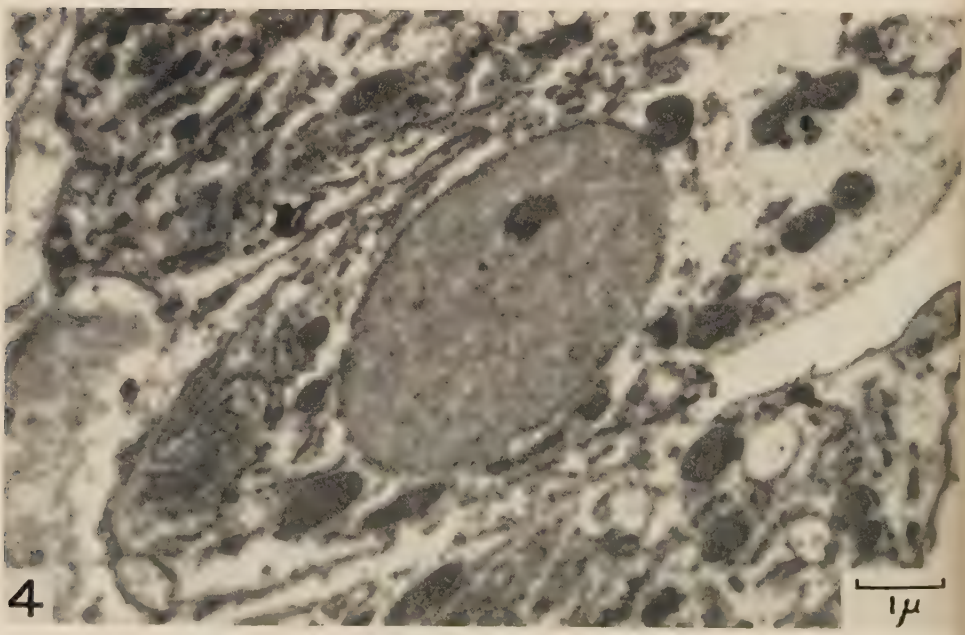

4
1μ

TABLE V

Measurements from electron micrographs at mag. × 20,000 of non-lactating alveolar cells.

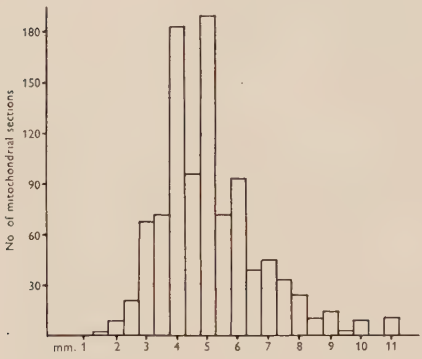
Total area of cytoplasm cm^2	Total area of sections of mitochondria cm^2	Number of sections of mitochondria	Total perimeter of sect. of mitochondria cm	B/A	C/A	D/A
A	B	C	D			
434.81	22.05	56	140.48	0.05	0.13	0.32
353.93	12.69	70	123.80	0.04	0.20	0.35
382.24	13.16	71	128.98	0.03	0.19	0.34
337.57	19.71	44	108.49	0.06	0.13	0.32
224.48	19.69	26	84.68	0.09	0.12	0.38
259.69	18.04	32	89.40	0.07	0.12	0.34
397.15	23.77	48	132.21	0.06	0.12	0.33
349.69	25.86	57	152.88	0.07	0.16	0.44
339.78	24.03	49	131.92	0.07	0.14	0.39
231.36	18.35	64	133.54	0.08	0.28	0.58
303.48	26.29	63	170.52	0.09	0.21	0.56
317.11	23.60	44	122.82	0.07	0.14	0.39
396.82	27.17	41	126.80	0.07	0.10	0.32
271.97	20.86	31	100.63	0.08	0.11	0.37
255.58	26.62	54	144.79	0.10	0.21	0.57
182.51	12.89	30	76.86	0.07	0.16	0.42
336.39	20.14	39	108.28	0.06	0.12	0.32
175.13	13.56	26	70.92	0.08	0.15	0.40
257.37	17.76	42	106.77	0.07	0.16	0.41
209.33	21.38	33	110.03	0.10	0.16	0.52
454.81	25.95	49	145.56	0.06	0.11	0.32
168.96	12.85	42	92.42	0.08	0.25	0.55
186.15	18.98	50	132.10	0.10	0.27	0.71
245.17	23.32	68	165.32	0.09	0.28	0.67
			Mean	0.07	0.17	0.43
			$s_{\bar{x}}$	± 0.004	± 0.012	± 0.024

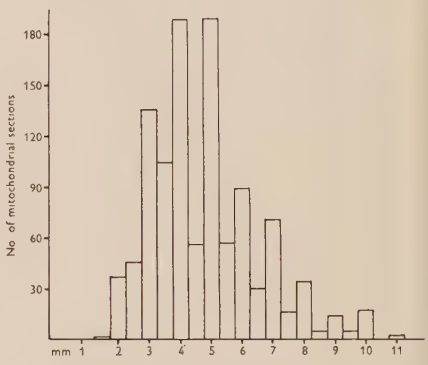
actating phase. Contrary to expectation the interface between the mitochondria and cytoplasm as given by the D/A ratios is greater in the non-actating cells.

ig. 3. Alveolar epithelial cells from non-lactating guinea-pig mammary gland in an electron icrograph. Buffered osmium tetroxide fixation.

ig. 4. Alveolar cells from a lactating guinea-pig mammary gland. Electron micrograph after uffered osmium tetroxide fixation.

It will be noted in Table III that the previous calculation from stained paraffin sections for the number of sectioned mitochondria per unit area of cytoplasm is much greater than that obtained from the electron micrographs. This is due to the respective magnifications. The values for C/A should be inversely proportional to the square of the magnification, and whilst there is fairly close agreement for the lactating series, the value for C/A for the





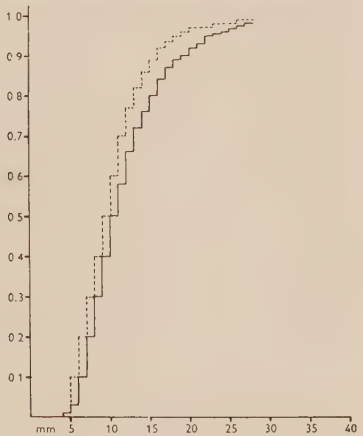
Text-fig. 1 (left). Histogram of short-axis measurements of sectioned mitochondria in electron micrographs, mag. × 20,000. Lactating series.
Text-fig. 2 (right). Histogram of short-axis measurements of sectioned mitochondria in electron micrographs, mag. × 20,000. Non-lactating series.

non-lactating series estimated from stained sections would appear to be too low. This may well be due to inaccurate identification and counting of the small mitochondria which are more numerous in the non-lactating material.

The measurement of the axes of approximately 1000 sectioned mitochondria in each of the two phases of the mammary alveolar cell has prompted us to enquire whether the mitochondria are of uniform diameter, varying only in numbers and in length or, if not, whether measurement of diameter is likely to be useful in distinguishing between the two different functional states under consideration.

It is clear from the stained preparations that most of the mitochondria are elongated, cylindrical bodies ranging in length to a maximum which appears to be about 10 times the diameter. The longer filaments are seldom straight and may be sufficiently curved to be cut more than once in a thin random section. It becomes, therefore, much more difficult to be precise about their true dimensions in electron micrographs than is the case with mitochondria which are roughly spherical or ellipsoidal as in some other types of cells. The majority of the filaments will be cut obliquely, giving an elliptical section,

the short axis of which will be equal to the diameter of the filament, except in the case of tangential fragments not passing through the central axis. With sections 0.05μ in thickness, it is possible theoretically to obtain about 6 sections parallel to the axis of a perfectly straight mitochondrion of diameter from about 0.25–0.3μ. Four of the sections in this case would have a width less than the diameter of the mitochondrion. Kinking would increase the



Text-fig. 3. Cumulative relative frequency distribution of the long-axes of sections of mitochondria from electron micrographs, mag. × 20,000. Full line = lactating series; broken line = non-lactating series.

number of possible sections through an individual mitochondrion, at the same time decreasing the proportion of oblique slices not passing through the central axis. As the plane of section is tilted with reference to the long axis, the tangential slices or fragments become reduced to a small proportion of the total. Since the orientation of the filaments is unpredictable with reference to the plane of section, the mitochondrial sections in electron micrographs form a very mixed population consisting mainly of oblique and tangential slices. It might be assumed that the latter, which have a short axis less than the true diameter, would form only a very small proportion of the total. However, in the three models containing plaster mitochondria all of the same diameter, but not kinked and curved to the same degree as in the actual cells, as many as 25 per cent had been cut as fragments narrower than the known diameter. Incidentally there was almost as much variation in the shapes of the sectioned mitochondria in the models as could be found in the electron micrographs.

The histograms in Text-figs. 1 and 2 giving the frequencies of the short-axis measurements respectively for the lactating and non-lactating material show first of all that the mitochondria cannot be of even diameter. There is a range of possible widths extending from about 3–11 mm in the electron

micrographs, which corresponds to an actual size of 0.15–0.55μ. In all probability the majority lie within the 0.2–0.25μ range but it is clearly impossible to sort out a distribution of this kind with any accuracy. The histograms for the lactating and non-lactating material are very similar, but by plotting the cumulative relative frequency distribution of these values it can be shown both by the Kolmogorov-Smirnov and the χ^2 tests that there is a significant difference between the lactating and non-lactating phases. The narrower mitochondria appear to be more numerous in the non-lactating series.

Although it is impossible from electron micrographs to get any measurements corresponding to the actual lengths of the mitochondrial filaments, a similar pair of histograms was used for the long-axes of the mitochondrial sections. These gave a cumulative relative frequency distribution as shown in Text-fig. 3, from which it can be seen that there is a consistent divergence between the values for the two phases of the gland. This confirms the qualitative evidence of the photomicrographs that there are more elongated filaments in the lactating phase but unfortunately it gives no real measure of the situation in the two cell-types.

DISCUSSION

The cytology of the guinea-pig mammary gland has been described quite frequently in the past possibly because this species presents the decided advantage over the rat and other laboratory animals in having a minimum of fat in the cytoplasm of its secretory cells, especially in the non-lactating stage. The early literature has been reviewed by Weatherford [21] and Jeffers [13]. It contains information which is entirely qualitative in character and difficult to appraise. No previous studies on the details of mammary epithelium appear to have been made employing perfusion fixation, and much of the information presented seems biassed in favour of showing that mitochondria play an active role in the formation of fat, or that they undergo extensive transformations during the secretory cycle. Working with electron micrographs and with models leads one to suspect that even the simple descriptions in previous papers of the relative amounts of granular and filamentous mitochondria appearing in different phases of the mammary cell may be inaccurate through confusion between oblique sections of filaments and complete rods or granules lying within the confines of individual sections. Unfortunately there are no photomicrographs in the early literature from which one can judge the quality of fixation. No attempt has been made in the present investigation to deal in detail with the various stages of the secretory cycle which, as is well

known, involve considerable alteration in the shapes of the alveolar cells. The change in shape is related to the filling of the alveolus emptied after suckling and may even involve distension if the secretion is not removed. It may well be that the mitochondria undergo extensive changes in form and substance during the elaboration of the secretion and its discharge from the cell. So far, the electron micrographs fail to reveal any internal structural alteration in the mitochondria during secretion. No indication of an apocrine disruption of the apical cytoplasm has been seen. This confirms the opinion already expressed (Richardson [18]) that cell decapitation is more likely to be associated with poor fixation, or with oblique sectioning of the tall columnar cells in collapsed alveoli.

Some general comment is necessary on measurement of mitochondrial diameters. If these bodies were spherical or ellipsoidal in shape, it would be possible to sort out their sections in electron micrographs to get a fairly accurate estimate of variation in true diameter. Unfortunately they do not appear to be always regular in cross section. Some have thickenings and excrescences and, when elongated, they are seldom straight. The ends of the filaments would seem to be hemispherical. If we take into consideration curvature and kinking and allow also for tangential slicing or sections passing through the hemispherical ends (a not uncommon occurrence in the models), it becomes impossible to do more than record a range of probable diameters. We would disagree, for instance, with the description given by Howatson and Ham [12] that in Fig. 4 of their paper the electron micrograph illustrates the considerable variation in diameter exhibited by the mitochondria". Dumb-bell contours such as that in Fig. 2 of the same paper, while suggesting fission, may be produced also by oblique sectioning through a curved mitochondrion.

One of the major obstacles at present hindering quantitative work on fixed cells lies in our inability to compute cell volumes accurately from sections. Hoffman [11] deals very fully with this problem and points out how the tendency still lingers for nucleo-cytoplasmic ratios, for instance, to be calculated from area measurements as though a valid comparison were being made of volumes. It is only in exceptional cases, in fact, where cells approximate closely to spheres, ellipsoids or cubes that three dimensional measurement is possible from sections. Probably the most elaborate attempt made from serial sections is that of Lecomte du Nouy and Cowdry [14] who measured pancreatic acinar cells including the dimensions of the mitochondria and zymogen granules in order to check a thermodynamic hypothesis of cell size. If one wished to measure hundreds of cells in order to allow for varia-

tion, such a method, even if sufficiently accurate, would be intolerable. In the work of Thurlow [20] who measured the number of mitochondria in sections of the neurones of the IX and XI cranial nerve nuclei in the mouse, the results are presented as the number of mitochondria per unit volume of cytoplasm. The mitochondria in these cells were granular in form and seemed to be evenly distributed. Although there was no statistical evaluation of the counts, the final conclusion that there was a constant number of mitochondria per unit volume of cytoplasm in normal nerve cells of a corresponding type, and that this constant differed for other types of nerve cells, remains as an interesting finding to be confirmed by some other approach.

The mammary cell presents special difficulties owing to its irregular shape. One might attempt to estimate cell volume by measuring groups of cells cut in planes approximately vertical and horizontal to the basement membrane, but since individual alveoli cannot be assumed to be in a comparable state of distension or collapse, such measurements of average cell size would still be rather unsatisfactory.

It is now common practice in biochemical investigations to count nuclei in a haemocytometer from homogenized tissue and to find the total number of nuclei in a known volume of tissue by estimating its DNA content. This approach (see Davidson and Leslie [8]) is based upon the work of Boivin, Vendrely and Vendrely [6] who showed that, with the exception of the germ cells, the mean amount of DNA per nucleus is apparently constant throughout the tissues of a given species. Allard *et al.* [2, 3] have combined estimations of the total number of cells in normal, regenerating and neoplastic rat livers with counts of isolated mitochondria from samples of homogenized tissue. Thus they have been able to show that the average normal liver cell contains about 2500 mitochondria. The neoplastic cell contains much less mitochondria. More recently Howatson and Ham [12] using electron micrographs of liver tumour sections have counted the average number of mitochondria per cell using estimations of average cell volume. Unfortunately these authors give no indication as to the method used for calculating cell volume and no statistical details of their counts. Nevertheless their results are in reasonable agreement with those of Allard *et al.* [3].

Before the biochemical methods are applied as a standard procedure to other tissues and organs, it must be realised that while they may have great value in comparisons of the overall mitochondrial content of an adult organ in normal, regenerating or pathological states, they do not necessarily give an accurate picture of the mitochondrial content of individual cell-types. As far as we are aware no notice has been taken in connection with the work

on liver of the fact that, according to Abercrombie and Harkness [1], only about 60 per cent of the nuclei in liver belong to the parenchymal cells. The mitochondrial content of the remaining 40 per cent of mixed non-secreting cells may indeed be relatively small enough to be ignored, or may be sufficiently constant in different normal and pathological states to be taken for granted.

At present, we have no idea of the relative proportions of stromal and parenchymal cells in the mammary gland, but it is obvious that considerable differences must exist between the cell populations in the non-lactating and lactating glands. Moreover, the non-lactating gland contains comparatively much more duct tissue per unit volume since the alveoli at this stage remain relatively unexpanded. It would appear then that quantitative work on the secretory cells of the mammary gland will require considerable preliminary investigation on the structural side and for this reason we believe the present results are of some value. Allowing for volume changes during fixation and embedding, which we can only presume to be the same for all the material examined under the electron microsope, it would appear that there is some constancy in the amount of mitochondrial substance per unit area of cytoplasm. The cells at the end of pregnancy contain a slightly larger number of mitochondria per unit area than will be the case when lactation is in progress, and many of the mitochondria are smaller in diameter and shorter than those in the lactating cell. In response to lactogenic hormones initiating and maintaining secretion, the cells increase in volume and coincidently the mitochondria elongate and some of them increase in diameter.

From the preliminary estimations on stained sections it would appear that the average number of mitochondria per cell undergoes a slightly more than twofold increase as the cells become actively secreting. This is not confirmed by the electron micrographs as far as the number of mitochondria per unit area of cytoplasm is concerned. Indeed the non-lactating appears to have significantly more than the lactating material. It has already been pointed out that the counts of mitochondria in the stained sections of non-lactating material appear to be too low. If this is the case, the average number per cell should be higher, but it is unlikely that any adjustment made by more accurate identification and better staining would bring the numbers per cell in the two phases into close approximation. One additional factor, however, must be taken into consideration. This is the elongation of the mitochondria occurring in the cell as it increases its volume, taken in conjunction with the thickness of the section. In three-dimensional models it is possible to show

that a slice at 2μ through a lactating cell with elongated mitochondria wil tend to include more sections of mitochondria than a similar slice through a non-lactating cell with the same number and even distribution of shor mitochondria, simply because there is some overlapping of the longer fila ments. Unless the filaments are strongly curved and kinked, a section a 0.05μ will give approximately the same counts in each type of cell, remember ing that the majority of mitochondria are cut transversely or obliquely to the long axis. It is probable, therefore, that in stained lactating cells the counts of mitochondria have been exaggerated by their change in form. Although there must be some increase in numbers as lactation is initiated, the evidence from sections remains conflicting. When biochemical studies on mammary epithelium are to hand, it will be interesting to see whether estimations based upon the "average mammary cell' will clarify this uncertainty. Great care will obviously have to be taken to ensure that the elongated filaments of the lactating cells are not fragmented during homogenization of the tissue. At present, it seems safe to visualize some increase in volume of mitochondrial substance per unit volume of cytoplasm in the lactation phase because the mitochondria increase in bulk, particularly in length. It is unlikely, however that there is a disporportionate increase due to crowding in excessive numbers since the maximum increase in numbers observed in the stained sections seems hardly enough for distribution throughout the increased volume of cytoplasm.

SUMMARY

Mitochondria have been measured in sections of mammary alveolar cells at the end of pregnancy and during lactation. The increase in respiratory metabolism characteristic of the lactation phase is accompanied by an hypertrophy of the cells, the average area of which in random sections is twice that of the non-lactating cells. However, the average area of mitochondrial substance per unit area of cytoplasm measured from electron micrographs remains about the same in the two phases of the cell. There are small increases in diameter of some of the mitochondria and their average length increases but, by inference, an increase in the number of mitochondria must be the principal change.

Any attempt to count the number of filamentous mitochondria from sections is liable to be inaccurate because some of them will be cut more than once in a section 0.05μ thick. Stained paraffin sections at 2μ give results which conflict with those from electron micrographs probably because there is

overlapping of filaments within the thickness of the section. According to experiments with models, an estimate of the number of mitochondria in an average alveolar cell could be obtained from the volume of the cytoplasm, the average diameter and average length of the mitochondria together with the average ratio of the area of mitochondrial substance to cytoplasm measured from electron micrographs. In computing the volume of mitochondria in a particular cell-type it would be necessary only to know the volume of the cytoplasm and the above ratio of areas obtained from electron micrographs. For cells of irregular shape the main difficulty lies in accurately determining the volume of the cytoplasm.

The relationship between measurements derived from sections and from isolation of mitochondria in homogenates of living tissue is discussed.

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THE NATURE OF THE "GHOSTS" OBTAINED BY LYSOZYME LYSIS OF *BACILLUS MEGATERIUM*

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When cells of *Bacillus megaterium* are treated with lysozyme in phosphate buffer, pH 7, two elements, visible in the phase contrast microscope, remain after completed lysis: granules and membrane-like "ghosts" [11]. The granules very probably consist of lipid material. This has been shown by analyses of material either extracted from whole cells [3] or obtained by differential centrifugations of cell lysates [12].

If the lysozyme treatment is performed in 0.1–0.2*M* sucrose, the bacterial cell wall is dissolved but the rest of the cell remains as an intact structural unit, the protoplast [11]. These protoplasts may be lysed by dilution of the solute. The same kind of granules and "ghosts" as described above are then obtained.

It is generally assumed that the bacterial protoplasm is surrounded by a so-called cytoplasmic membrane that determines the permeability properties of the cell. The existence of such a membrane in the form of a distinct morphological unit has not been unambiguously proved, but the presence of a permeability barrier at the surface of the protoplasm has been demonstrated [9, 6, 13].

The present author has suggested [11] that the "ghosts" mentioned above might represent cytoplasmic membranes, either intact or denatured. If the "ghosts" were equivalent to undamaged membranes, the following quantitative relationships should be expected between them and the protoplasts. First, a given number of protoplasts should give rise to the same number of "ghosts", i.e. a numerical ratio equal to one should exist between the two structures. Second, protoplasts should give rise to "ghosts" having, at least approximately, the same average size. Third, similar size distributions should be found in populations of protoplasts and "ghosts". Statistically, this would be manifested by similar standard deviations.

The relationships should hold even if the "ghosts" are prepared directly from whole cells, i.e. not via protoplasts, since very slight change, if any, takes place in the surface structure of the protoplasm when the cell wall is removed.

This is evidenced by the fact that the protoplasts respire at the same rate and exhibit similar permeability properties and synthetic biochemical capabilities as intact cells [2, 13, 5, 14, 10, 7].

Fig. 3*b*, reference [11], represents a photomicrograph of "ghosts" prepared by lysis of *B. megaterium*. It is evident that their size varies within rather wide limits. In addition, most of the lipid granules of the intact cells are found outside the "ghosts". That the "ghosts" usually are characterised by a fairly large variation in size is also shown by Fig. 2, reference [12].

Thus as judged from the experimental evidence collected so far, the identity of the "ghosts" with cytoplasmic membranes seems somewhat doubtful.

In the present paper it will be shown, however, that the quantitative relationships mentioned above exist, provided that the preparation of the "ghosts" is carried out under special conditions. Such "ghosts" are thus very probably built up essentially of material present at the surface of the bacterial protoplasm.

MATERIALS AND METHODS

B. megaterium, strain KM [8] was used in all experiments. Cultures for experimental work were grown in 2 per cent Difco peptone at 30° C with mechanical agitation using Erlenmeyer or Fernbach flasks. The cells were harvested during the phase of declining growth rate (8–12-hour cultures).

Electron microscopy.—A Siemens 90 kV electron microscope, working at a magnification of about 2000 diameters, was used. For counting purposes a polystyrene latex suspension [1] was added to the sample to be investigated (final concentration 3.38×10^{10} particles/ml, size of particles 0.26μ). For further details of the counting procedure, see Williams and Backus [15] and Luria, Williams and Backus [4].

Light microscopy.—Photomicrographs were taken with phase contrast at an original magnification of 1300 diameters, using an Ortholux microscope equipped with an apochromatic 90× oil immersion objective, N.A. 1.10, and a 8× eyepiece. Measurements of particles under the microscope were carried out with a Leitz ocular micrometer (12.5× eyepiece). Particle counting was carried out with the aid of a Bürker counting chamber. A 10× objective was used in this case and dark field illumination was applied. For details see Weibull [13].

Analytical methods.—Nitrogen was determined according to Kjeldahl. Ultraviolet absorption measurements were made with a Beckman model DU spectrophotometer.

EXPERIMENTAL RESULTS

The starting point of this investigation was the observation that magnesium ions stabilise the protoplasts of *B. megaterium*. As shown earlier, these bodies are stable in 0.2*M* sucrose when kept semianaerobically, but disintegrate if the

suspension is shaken with air. Magnesium salts at a concentration of 0.005M prevent this kind of disintegration. Simultaneously, the respiration of the protoplasts, as measured by Warburg experiments, is strongly inhibited. When the sucrose is removed, e.g. by dialysis, the protoplasts disintegrate even in the presence of magnesium ions, giving rise to "ghosts".

As mentioned above, the size of normal "ghosts" (i.e. prepared in the absence of magnesium ions) varies within rather wide limits. As an average the "ghosts" are smaller than the corresponding protoplasts. In addition to spherical forms often non-spherical membranes are found in the lysates, together with free lipid granules. The "ghosts" obtained after the protoplasts have been in contact with 0.005–0.01M magnesium sulphate or chloride for 4–8 hours look quite different (Fig. 1*b*). They are practically all spherical and have about the same diameter as the corresponding protoplasts (Fig. 1*a*). Rather few lipid granules are found outside the "ghosts".

The experimental details for preparing the latter type of "ghosts" were as follows. To each 1 ml sample of a bacterial suspension containing *c.* 30 mg bacterial dry weight/ml (suspending medium 0.02M phosphate, pH 7.0), 0.2 ml 1M sucrose and 0.1 ml 1 per cent lysozyme was added. When the cells had been converted into protoplasts, 0.1 ml 0.13M magnesium chloride was added and the suspension was allowed to stand for 8 hours at room temperature. The "ghosts" were then obtained by diluting the protoplast suspensions with 2 ml 0.01M magnesium chloride and water to 10 ml.

Table I gives the results of the measurements carried out using the light microscope. The diameter of 100 unfixed protoplasts and "ghosts" were measured. Before counting, protoplasts were fixed in 4 per cent formaldehyde. "Ghosts" prepared in the presence of magnesium ions could readily be counted. Preparations made in the absence of these ions, on the other hand, were not well suited for counting experiments because of the small size and weak contrast of the "ghosts" obtained. The number of protoplasts and

TABLE I

Number and size of protoplasts and "ghosts" as determined in the light microscope. The "ghosts" were prepared in the presence of magnesium ions. The numbers refer to 1 ml original bacterial suspension, containing *c.* 30 mg bacterial dry weight/ml.

	Number	Average diameter, μ	Stand. deviation of diameter, μ
Protoplasts	$(2.12 \pm 0.03) \times 10^{10}$	1.64 ± 0.02	0.186 ± 0.013
"Ghosts"	$(2.08 \pm 0.03) \times 10^{10}$	1.87 ± 0.02	0.178 ± 0.013

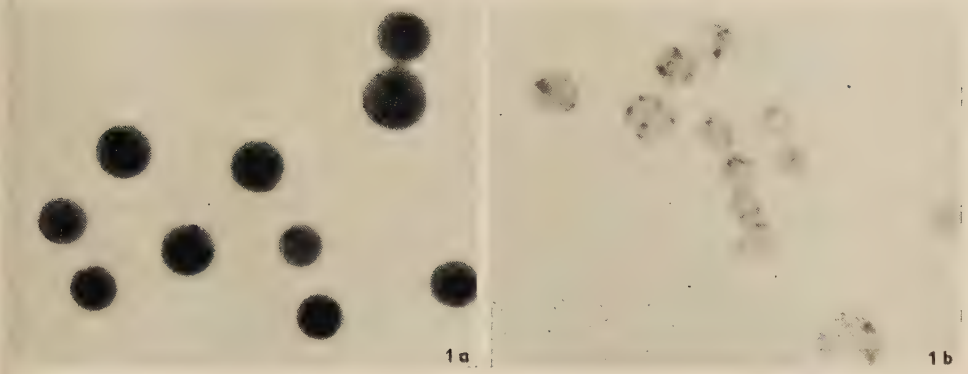


Fig. 1. Photomicrographs of protoplasts and "ghosts" of *B. megaterium*, 3000 ×. The photographs were taken, developed and printed under identical conditions. *a*, protoplasts; *b*, "ghosts" obtained by lysis of protoplasts in the presence of magnesium ions.

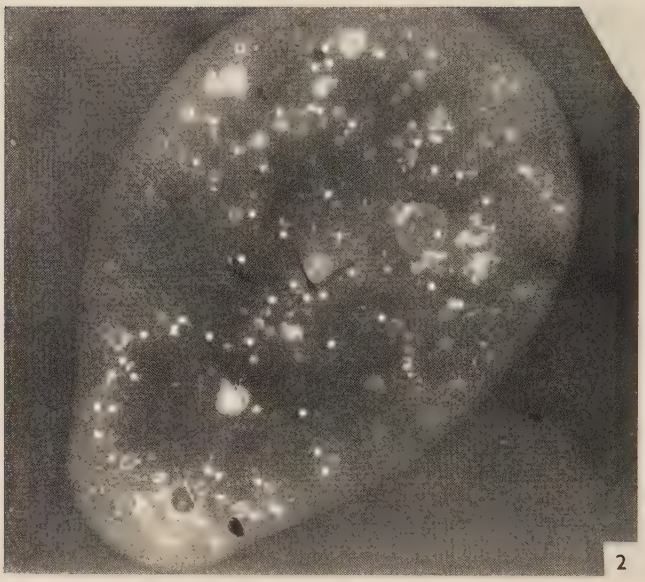
"ghosts" given in Table I refer to 1 ml of the original bacterial suspension and are thus directly comparable.

The number and size of "ghosts" of different types were also determined using the electron microscope. The figures obtained were compared with the number of the corresponding protoplasts, as determined with the aid of the Bürker counting chamber. "Ghosts" were prepared by direct lysis of whole cells or by lysis of protoplasts either in the absence or in the presence of magnesium ions. The same amounts of bacterial suspension, sucrose and lysozyme were used as in the experiments with the light microscope. Before spraying the experimental suspensions of the specimen grids, the samples were dialysed for 4 hours (longer dialysis, as a rule, caused aggregation

TABLE II

Number and size of protoplasts and "ghosts". The protoplasts were counted with the aid of a Bürker counting chamber, the "ghosts" with the electron microscope. The "ghosts" were prepared in the following ways: by direct lysis of cells (I), by lysis of protoplasts either in the absence (II) or in the presence (III) of magnesium ions. Two independent experiments were performed (a and b).

	Number $\times 10^{-10}$		Ratio "ghosts"/protopl.		Size, μ
	a	b	a	b	a, b
Protoplasts	2.18 ± 0.03	4.34 ± 0.09			
"Ghosts" I	7.73 ± 0.37	14.8 ± 0.6	3.54	3.40	0.2–1.2
"Ghosts" II	6.22 ± 0.23	16.4 ± 0.9	2.85	3.78	0.2–1.0
"Ghosts" III	2.92 ± 0.17	5.70 ± 0.33	1.34	1.31	1.4–2.5

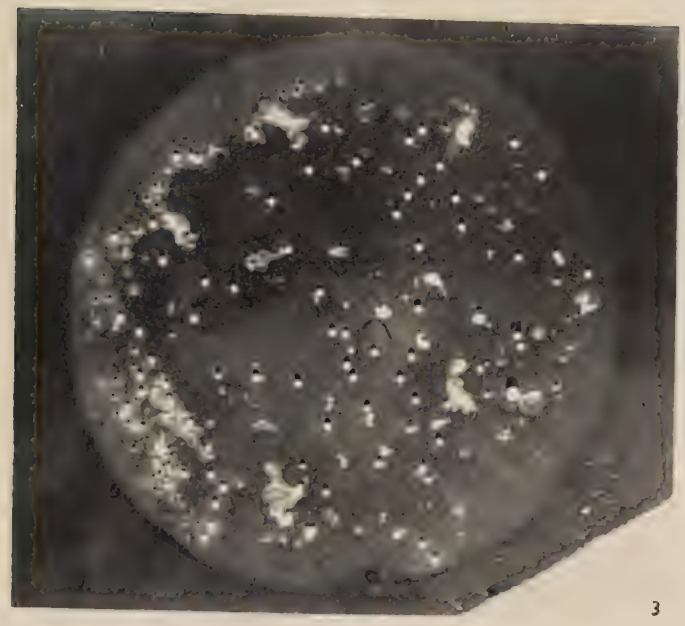


Figs. 2–4. Electron micrographs of bacterial lysates mixed with a polystyrene latex suspension and sprayed on specimen grids, *c.* 3000×. 2. Intact cells lysed in the absence of magnesium ions. 3. Protoplasts lysed in the absence of the same ions. 4. Protoplasts lysed in the presence of magnesium ions.

phenomena). The "ghosts" prepared in the absence of magnesium ions were fixed for 30 minutes in osmic tetroxide vapour. Table II gives the results obtained from two independent experiments. As in the experiments previously described, the figures are directly comparable.

Figs. 2–4 represent electron micrographs of droplets formed by sprayed "ghost" suspensions. Fig. 2 shows "ghosts" obtained by direct lysis of cells, Fig. 3 the same structures remaining after lysis of protoplasts in the absence of magnesium ions. Fig. 4 shows "ghosts" from protoplasts that have been in contact with magnesium chloride. In all electron micrographs, a fairly large amount of background material, essentially conisting of bacterial proteins, is present in the droplets. In Figs. 2 and 3, many free lipid granules can be observed.

It could be supposed that magnesium ions would influence the amount of cytoplasmic material released from the protoplasts at the moment of their lysis. Therefore, lysates were analysed for nitrogen and ultraviolet-absorbing material after removing the "ghosts" by centrifugation at 15,000 *g* for 15 minutes. Table III gives the results. After some hours, a turbidity appeared in the centrifuged solutions that contained magnesium ions.



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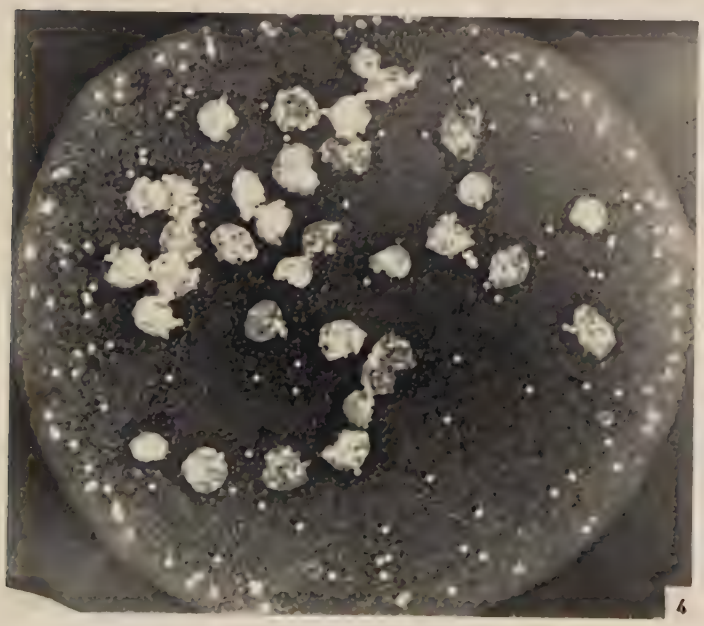

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TABLE III

Nitrogen content and ultraviolet absorption of bacterial lysates after removal of "ghosts" by centrifugation. The lysis took place either in the presence or in the absence of magnesium ions. In all other respects the lysates were identical.

	Nitrogen-content mg/ml	E_{260} (0.1N NaOH)
0.01M magnesium chloride present	0.650	20.3
No magnesium ions present .	0.748	22.5

The "ghosts" of *B. megaterium* described earlier have been shown to contain most of the pigmented material of the intact cells [12]. This is also true when they are prepared in the presence of magnesium ions.

DISCUSSION

The appearance of "ghosts" prepared in the presence of magnesium ions suggests that they represent protoplasts deprived of their contents (cf. Fig. 1*a* and *b*).

Table I, which summarizes the numerical determinations carried out by use of the light microscope, shows that, within a few per cent, a given number of protoplasts gives rise to an identical number of "ghosts" under the specified conditions. The "ghosts" are, on an average, slightly larger than the protoplasts. Some stretching phenomena may, however, very likely occur when the protoplasts are lysed. The size distribution of the "ghosts" and of the protoplasts is, within the statistical error limits, similar. This is evidenced by the respective standard deviations.

The results from the counting experiments carried out with the electron microscope (Table II) are characterised, *inter alia*, by a certain excess of "ghosts" over protoplasts, amounting to about 35 per cent when the "ghosts" are prepared in the presence of magnesium ions. However, some of the membrane-like structures present in Fig. 4 give the impression of being "ghosts" split into halves. The splitting probably occurs during the spraying process. A "ghost"/protoplast ratio of 1.3–1.4 instead of 1.0 could thus be explained.

In the absence of magnesium ions, the ratio is about 3–4, i.e. far from unity. The size variation is considerable in comparison to the rather uniform size of the "ghosts" prepared in the presence of magnesium ions.

It thus seems that the "ghosts" prepared in the presence of magnesium ions represent morphologically unchanged cytoplasmic membranes. Small

amounts of protoplasmic material may still be present within, or absorbed to, the membranes.

The centrifugation experiments (Table III) indicate that, after removing the "ghosts", somewhat less cytoplasmic material is left in the supernatant solution in the presence than in the absence of magnesium ions. The excess material centrifuged down may not necessarily be directly related to the "ghosts", since in the former case a slight amount of white precipitate was noted on top of the yellow "ghost" layer.

Whether or not the membranes obtained have become more or less denatured, in the chemical sense of the word, must remain an open question. The strong inhibition of the respiration of the protoplasts in the presence of magnesium ions makes denaturing phenomena not improbable. In order to settle this question, enzymic and permeability experiments of "ghosts" and protoplasts of different types would be desirable.

SUMMARY

"Ghosts" prepared by lysozyme lysis of *Bacillus megaterium* cells have been investigated. The numerical ratio "ghosts"/protoplasts and the average size of the "ghosts" together with the size distribution indicate that the "ghosts" under certain conditions represent morphologically undamaged cytoplasmic membranes.

The author wishes to thank Professor A. Tiselius for his kind interest in this work. The author is very grateful to Dr. R. C. Williams, The Virus Laboratory, Berkeley, California, for the polystyrene latex suspension used in this investigation.

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BRIEF NOTES

ON THE DISTRIBUTION OF NUCLEIC ACIDS IN THE ROOT TIP OF *VICIA FABA*

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In recent years a large amount of evidence has accumulated [1] indicating the importance of nucleic acids in the growth of the cell. The root tip offers a particularly useful material for studies in cell development as the stages of growth are clearly definable and easily analyzed. The purpose of the present investigation is to determine the distribution of desoxyribose nucleic acid (DNA) and ribose nucleic acid (RNA) per cell in the first three millimeters of the root tip of *Vicia faba* var. broad Windsor by the analysis of 200 micron sections. This area of the root tip has been previously analyzed in detail both for cell structure and several biochemical constituents [3]. It is, therefore, of interest to analyze the same material for nucleic acids.

Methods.—The tips of primary roots between 3–8 cm in length of the broad bean, *Vicia faba* var. broad Windsor, were used in all determinations. The method of culture, selection, and sectioning of the roots has been previously described in detail [3]. Only the first fifteen 200 micron sections, starting at the tip, were used.

The nucleic acid content of the sections was determined using a microadaption of the Ogar and Rosen procedure as modified by Klein *et al.* [4]. This procedure is as follows: (1) Prepare and section roots (3–5 roots used per series); (2) add 95 per cent ethanol and drain; (3) add ethanol-ether (1 : 3) and heat to 60°C for 10 minutes; (4) drain and chill to 0°C and keep in chilled aluminum block; (5) add 2 per cent perchloric acid (cold), agitate, and drain at once; (6) add 10 per cent perchloric acid (cold) 0.2 ml, leave overnight in refrigerator, save extract; (7) add fresh 10 per cent perchloric acid, leave in refrigerator 4 hours, save extract and add to 6; (8) wash residue with 5 per cent perchloric acid; (9) add fresh 5 per cent perchloric acid 0.2 ml and heat at 70°C for 20 minutes, save extract; (10) add fresh 5 per cent perchloric acid 0.2 ml, repeat step 9, save extract and add to 8.

The absorption of the extracts at 260 mμ was measured in a Beckman spectrophotometer model DU equipped with microcuvettes. Six sets of determinations were made.

For five selected areas complete absorption curves were made from 220 mμ to 300 mμ. The sections for this were taken 300, 500, 900, 1500, 2500 microns from the

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tip. In all cases there was a well defined peak at 260 264 mμ. This was very small, however, in the case of the 300 micron sample.

Results: (*a*) *Morphological.*—The first 3 millimeters of the primary root of *V. faba* has previously been subjected to a detailed cellular analysis [3]. Although these results will not be repeated here, the general histology of the root tip is presented in the longitudinal drawing in Fig. 1.

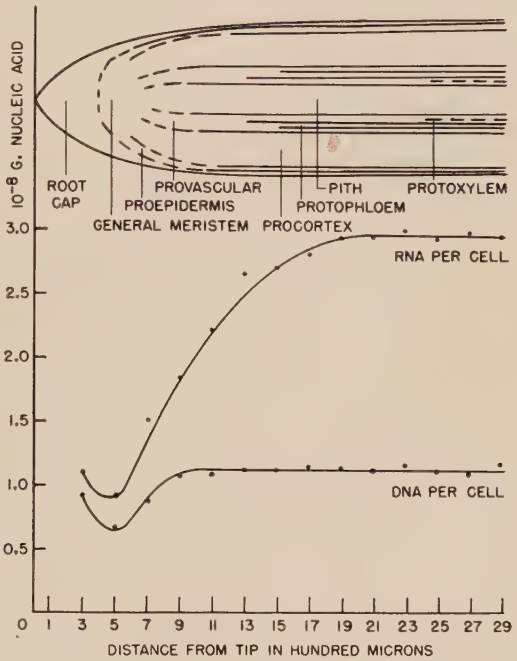

Fig. 1. The amount of RNA and DNA per cell as a function of distance of section analyzed from the tip of the root. The diagram of the root is on the small scale as the graph.

Several points concerning the morphology of the area must be emphasized:

1. This area (0–3 mm) of the root is highly complex and well organized. Except for the area of the general meristem (approx. 50 μ wide) the cells can be identified on the basis of the mature tissue system which they will compose. A majority of cell divisions occur in this region, but cell elongation, and primary cell differentiation are concomitantly occurring making it impossible to characterize the entire area as "meristematic."

2. Cell elongation in most cell types begins at approx. 1700 microns from the tip although the central or pith cells begin elongation at approximately 800 microns. Some limited elongation is occurring prior to these times associated with cell division.

3. Between the general meristem and ~1700 micron primary cell differentiation is occurring, manifest in cell shape primarily through increase in cross sectional area. Primary differentiation is used here in the sense that the various mature tissue systems are definable although few, if any, distinct mature cell types can be seen.

(*b*) *Nucleic acids.*—The results of the nucleic acid determinations are presented in Fig. 1. The results are expressed in 10^{-8} g per cell. The per cell value was obtained by dividing the section value by the number of cells per section. The cell numbers were those previously determined [3].

The reproducibility of the values is satisfactory. The DNA values for six sets of determinations varied by less than 3 per cent while the RNA values varied by less than 10 per cent.

Discussion.—There are several possible interpretations of the DNA results. Until, however, the exact number of cell divisions per section is known and per nuclei DNA determinations are made directly it will be impossible to present a definite interpretation of the data. It is interesting, nonetheless, that the cell of the general meristem has almost exactly half the amount of the more basal cells. The increase in DNA per cells correlates very closely with the area of primary cell differentiation.

The RNA results present quite a different picture. The RNA value per cell in the general meristem is quite low but during the stages of radial enlargement and primary differentiation the per cell value increases nearly four times until it becomes constant per cell at the beginning of cell elongation. This increase in RNA corresponds very well with the area of primary differentiation, although it is impossible to make a direct correlation as both cell division and limited elongation are occurring. It would appear of interest, however, that exactly where cell elongation is commencing the RNA value becomes constant.

When protein nitrogen determinations are made on similar sections the amount of protein per cell directly parallels the increase of RNA content per cell. Where the RNA per cell becomes constant, the protein value per cell decreases. The parallel rise in RNA and protein is interesting as it confirms in root tips what has been observed in many other organisms.

Recently, Holmes *et al.* [2] measured the nucleic acid content of 2 and 4 mm sections of the root of *V. faba.* They discarded the root cap thus making the first 2 mm section they analyzed comparable to the entire area analyzed here. They considered this area of the root as being completely meristematic and that elongation and differentiation occurred in more basal areas. This is in complete disagreement with the data presented here and elsewhere. So far as the nucleic acid content per cell is comparable, i.e. the RNA : DNA ratio, theirs is in rather good agreement. They feel however that there is a parallel rise in DNA and RNA with differentiation in more basal sections. While this may be true they miss the striking correlation of these two factors in the region of primary differentiation because the sections they analyzed were too large.

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EXPERIMENTALLY INDUCED CHANGES IN MITOCHONDRIAL MORPHOLOGY: VITAMIN A DEFICIENCY

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The comparatively limited resolving power of the light microscope restricted Wolbach and Howe in their classical studies on the pathology of vitamin deficiency states to descriptions of changes at cellular levels. However, they did attempt to determine whether there were any visible alterations in mitochondria associated with the keratinizing epithelia which appears in vitamin A deficient animals. Their observations led them to conclude that "the mitochondrial apparatus is not primarily affected in fat-soluble A deficiency" [6].

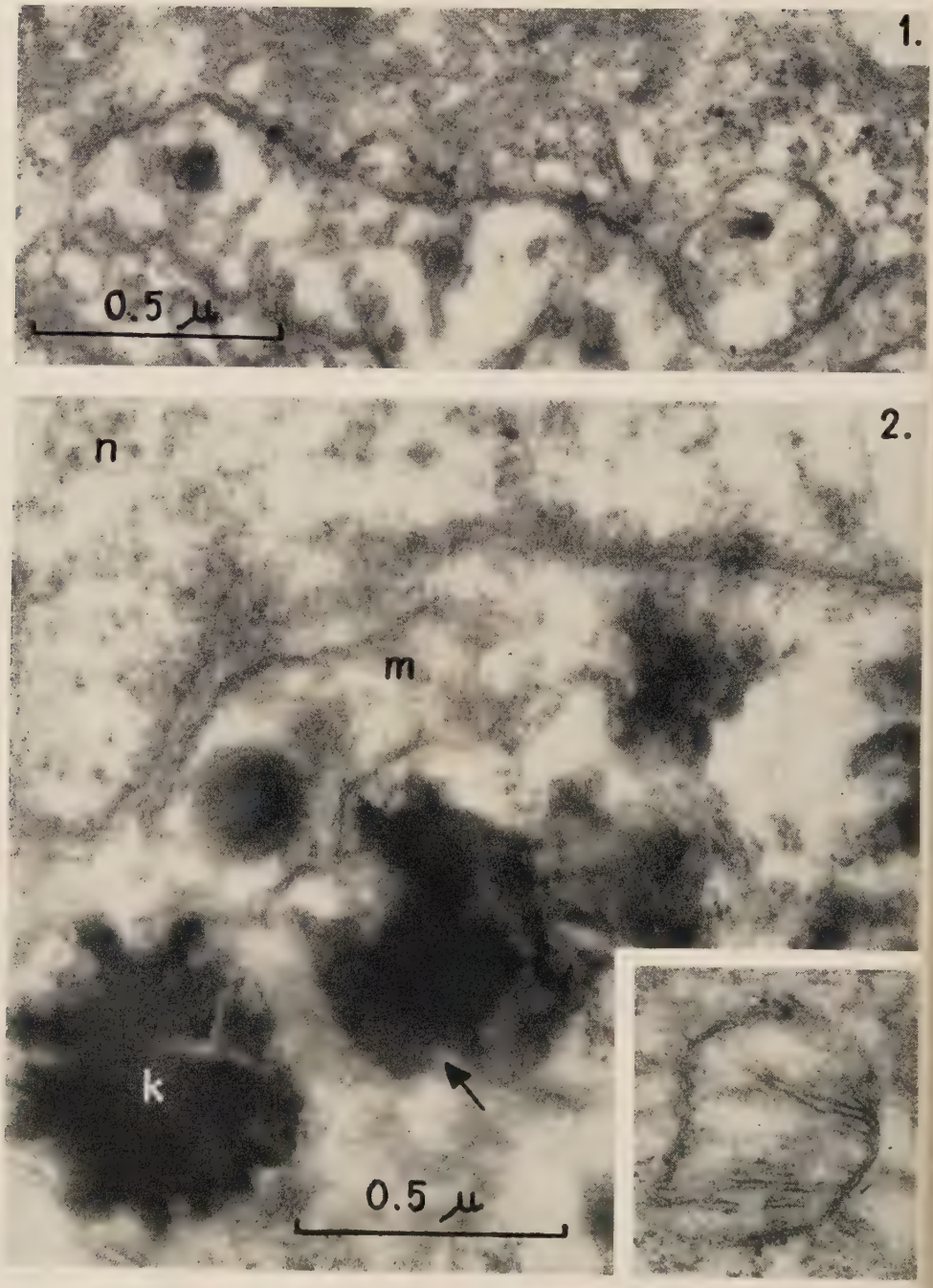
An electron microscope study of the corneal epithelium in the vitamin A deficient mouse indicates that changes in the appearance of the mitochondria coincide with the appearance of the so-called "keratohyaline bodies" in cells of the keratinizing epithelium.

This study was made on specimens taken at various times up to 250 days from mice on a vitamin A deficient diet. The diet was developed under the direction of Professor E. Brunius at the Vitamin Department of the Swedish National Public Health Institute. The experimental animals presented loss of weight, severe ophthalmia, ruffled fur, hunchbacked posture, listless behavior, and stunted growth compared to control animals on the same diet supplemented with vitamin A.

Within 90 seconds of sacrificing a clinically ill animal, the tissue was immersed in 1 per cent isotonic osmium tetroxide, dehydrated in graded alcohols and embedded in methacrylate. Sections were cut with a Sjöstrand microtome, mounted on copper grids, and examined in an RCA EMU 2c microscope [3].

Light and electron microscopy on the corneal epithelium of 15 animals demonstrated keratinization of the superficial layers as histologic evidence of avitaminosis A. There appeared to be decreasing amounts of chromatin in the nuclei of cells nearer the surface of the epithelium while in the most superficial layers nuclei could no longer be identified. However, dense granules about 1 2 microns in diameter could be distinguished in this keratinizing epithelium with the light microscope.

The electron microscope showed that the basal cells of the corneal epithelium contained many large mitochondria whose appearance did not differ radically from that of mitochondria described in other cell types and in the normal cornea of the mature mouse [2]. Mitochondria appeared as membranous systems with two parallel dense lines bounding transversly oriented parallel dense lines in both the normal cornea of the mature mouse and the basal cells of the keratinizing epithelium. These mitochondria did not contain the small dense granules peculiar to mitochondria of the proximal tubule kidney cells, acinar cells of the pancreas, and columnar absorbing cells of the intestinal epithelium [4, 5, 7].


1.
0.5 μ
2.
n
m
k
0.5 μ

Cells of the intermediate strata of the keratinizing epithelium in which Mori demonstrated the "keratohyaline bodies" first appear [1], showed many mitochondria whose internal structure was somewhat distorted. Apart from an apparent disorganization of many internal membranes a striking feature of these mitochondria was the appearance of one or more heavily dense areas within the mitochondrial matrix. These heavily dense areas were of different sizes in many different mitochondria and most often appeared to lie between the two dense components of the transverse mitochondrial membranes. Near the nucleus in most intermediate cells large homogenously dense granules similar in appearance to the small intramitochondrial dense bodies were seen. These large dense granules were often circumscribed at least in part by two parallel dense lines like the membrane bounding the "normal" mitochondria. These large dense granules lying near the nucleus were interpreted as the "keratohyaline granules" seen with the light microscope. While the Golgi apparatus of intermediate cells was often seen in close relation to the nucleus and to the large dense granules or "keratohyaline bodies", electron microscopy did not indicate an invariable topographic relationship.

Cells of the most superficial keratinized layers showed few mitochondria or any structures resembling these systems of membranes, though many large dense granules were apparent in the cellular skeletons.

It is most tempting to interpret the changes described above as representing the sequence of events following on the absence of vitamin A and to suggest that avitaminosis A alters the metabolic pattern of the mitochondria resulting in the accumulation of the dense substance which has hitherto been referred to as "keratohyaline".

We may at least conclude that the deprivation of vitamin A is in some way connected with the appearance of the keratinizing epithelium within which the so-called "keratohyaline granules" appear. In the intermediate cells where these granules first appear they are seen most often at the periphery of the nucleus. This study has shown that there are alterations in mitochondrial morphology which appear in conjunction with these changes. Whether the large dense granules actually stem from interference with normal mitochondrial metabolism may have to be demonstrated by other biochemical or biophysical methods.

The authors would like to express their appreciation to Dr. F. S. Sjöstrand for his interest and for the privilege of working in his laboratory and to Professor E. Brunius for his help and advice concerning the diets of the experimental animals.

Fig. 1. Electronmicrograph of longitudinal and cross-sectioned mitochondria from keratinizing corneal epithelium intermediate cell of vitamin A deficient mouse showing small intramitochondrial dense bodies between the dense components of the transversly oriented membranes. Magnification 67,000 ×.

Fig. 2. Electronmicrograph from similar specimen showing portion of nucleus (*n*), mitochondria (*m*) containing larger dense body and "keratohyaline" bodies (*k*). Arrow indicates double-contoured membrane. Magnification 74,000 ×.

Inset showing normal mitochondria. Magnification 79,000 ×.

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EFFECT OF STORAGE ON THE ENZYME ACTIVITY OF PARAFFIN-EMBEDDED TISSUE[1]

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In the application of histochemical procedures to tissues which have been embedded in paraffin it may be necessary to perform these techniques on blocks which were embedded some time previously. It is therefore desirable to know the effect of "storage" on the content of the particular substances to be determined. There are conflicting reports concerning the retention of enzymatic activity in stored paraffin blocks of tissue. Gomori [5] considers that alkaline phosphatase activity is retained unchanged in paraffin blocks for over 10 years, Lison [7] is quoted as finding this enzyme to deteriorate in two months, and Doyle [3] has also reported loss of this activity in stored paraffin sections. For acid phosphatase it has been reported by some [3, 4, 6] that activity gradually disappeared on storage of blocks, ribbons, and mounted sections, while others [2] report retention of activity for as long as 12 months or more in uncut blocks.

In order to obtain definitive data with regard to the stability of enzymes in stored, uncut paraffin blocks, a series of experiments were carried out on acetone fixed rat liver, which was analyzed histochemically for acid and alkaline phosphatase [5] and esterase [5] and quantitatively by homogenate techniques for acid and alkaline phosphatase [10], peptidase [1, 9], and esterase [8].

The livers of male, Sprague-Dawley rats fasted 18 hours were removed after exsanguination under ether anesthesia. Weighed samples 1 mm in thickness were obtained for immediate analysis and for fixation in acetone and embedding in paraffin. For immediate chemical analysis 10 per cent homogenates were prepared in ice cold isotonic saline and appropriate aliquots taken for determination of the enzymes

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mentioned above. Fixation in acetone was carried out at 4°C, the acetone being changed after approximately 1, 4, 8 and 24 hours. After the last change of acetone the liver samples were brought to room temperature for $^1/_2$ hour, cleared in 2 changes of chloroform in 1 hour and finally embedded in paraffin at 56°C for 1 hour. The paraffin blocks were stored at 4°C for periods of 1, 2, 3, 6 and 12 months. Promptly following embedding one sample of fixed liver was cut into sections at 25 mμ and the

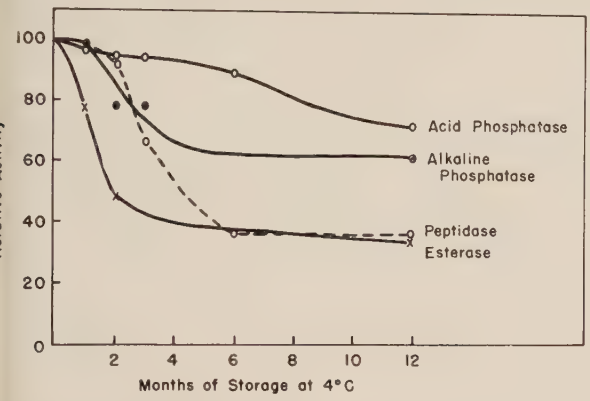

Fig. 1. Changes in relative enzyme activity upon storage.

paraffin was removed with 3 washes of xylene, centrifuging and decanting after each wash. The residual xylene was removed at room temperature under reduced pressure, the sample was homogenized to a tissue concentration of 10 per cent based on its initial fresh weight and assayed as above. The stored samples were processed in the same manner for analysis. Three sets of tissues were analyzed in this manner for each interval studied.

For histochemical study rat liver fixed in acetone and embedded in paraffin was cut at 6 mμ, deparaffinized with 2 changes of xylene and hydrated with a graded series of acetone-water solutions. They were subsequently examined for acid and alkaline phosphatase and esterase as previously mentioned. Histochemically, it was not possible to detect reduction of enzyme activity in paraffin blocks stored at 4°C for up to 6 months. After 12 months, however, some uneven staining and decreased activity were noticed.

The process of fixation in acetone and infiltration and embedding with paraffin as employed in these experiments gave a residual activity, as compared with fresh tissue of approximately 60 per cent for acid phosphatase, 65 per cent for alkaline phosphatase, 30 per cent for peptidase and 65 per cent for esterase. Following storage of such tissue at 4°C, further reduction of the content of these enzymes was observed as shown in Fig. 1, occurring most rapidly for peptidase and esterase, less rapidly for alkaline phosphatase, and slowly for acid phosphatase.

In two months at 4°C esterase activity decreased to approximately 50 per cent of that shown by paraffin blocks deparaffinized immediately after embedding, and to 25 per cent in 12 months. Peptidase activity dropped to 70 per cent in three months and to 35 per cent in 12 months. Similarly alkaline phosphatase decreased to 80 per cent in three months and to 60 per cent in 12, while acid phosphatase decreased

only slightly, to 95 per cent in three months, and to 70 per cent in 12 months. By 12 months of storage at 4°C the loss of activity in paraffin blocks for the four enzymes studied has slowed to an insignificant rate, whereas prior to this time considerable loss of enzyme may occur. The rate of loss is not the same for all enzymes.

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ON THE SULPHATE METABOLISM OF THE MOUSE BRAIN

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In the last five years our knowledge of the sulphate metabolism has been considerably increased through the use of tracer methods. Comparatively little, however, is at present known about the sulphate metabolism of nervous tissue. Incorporation of radiosulphate into grey matter of rat brains [4] and into the developing central nervous system of rat embryos [7, 8] has been reported. Radiosulphate is known to be incorporated into sulpho-mucopolysaccharides [6, 2] and sulpho-cerebrosides [10], whereas only inconsiderable amounts of isotope can be recovered from cystine, methionine and taurine [3].

The aim of the present investigation is to study the fate of labelled sulphate in the central nervous system of adult mice.

Experimental.—Intraperitoneal injections of 3μc/g body weight of a carrier-free radiosulphate solution were given to 176 mice with an average weight of 22 g. 17–18 animals were killed at 1.5, 3, 6, ... 768 hours after injections (cf. Table I). The brains were removed and blood samples were taken from the heart. The brains and the blood were dried to constant weight at 40°C, pooled and homogenized to a dry powder. The radioactivity was measured with a TGC-2 Tracerlab G-M tube on pooled dry powder samples of infinite thickness.

Another 48 mice obtained 8 μc/g body weight radiosulphate and were used for autoradiography. The animals were killed at the same intervals as the first series.

TABLE I

Radioactivity in brain and heart blood at different intervals after radiosulphate injection. C.P.M./cm².

Time hours	1.5	3	6	12	24	48	96	192	384	768
Brain	280	286	247	192	112	71	55	35	36	22
Blood	4891	4045	3050	1271	506	85	24	1	3	0

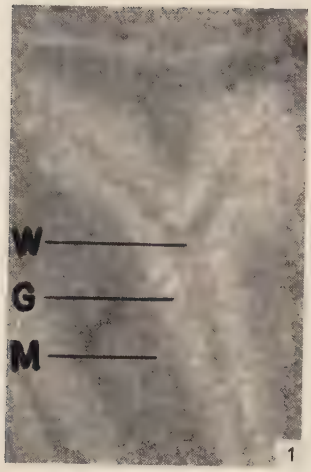
Two brains from each group were frozen sectioned. The remaining brains were fixed in methyl alcohol for 24 hours, then taken through ethyl alcohol and xylol, embedded in paraffin, sectioned, deparaffinized and dried. Autoradiography was then done as described elsewhere [8]. For comparison with the autoradiographs, sections were stained with PAS according to a modification of the Hotchkiss procedure involving the use of alcoholic solutions [8]. Other sections were stained with toluidine blue.

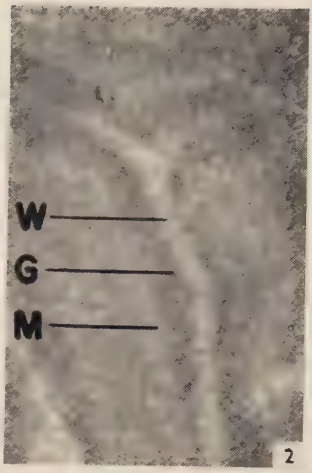
Results.—The maximum radiosulphate concentration in blood occurred at or before 1.5 hours after injection. After 1.5 hours the blood concentration rapidly decreased, being very low in the blood of animals killed after 48 hours. Three hours after injection the maximum radiosulphate concentration in brain was attained. The amount of isotope in brain then decreased rapidly during the following 45 hours. The decrease after 48 hours was considerably slower.

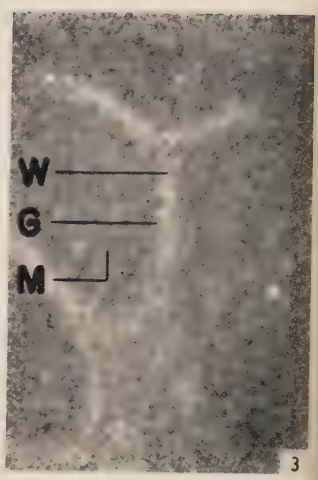
Autoradiographs from frozen sections of brain 1.5–6 hours after injection showed a rather even radiosulphate distribution. However, certain cell layers, characterized by a large number of cell nuclei, gave a stronger blackening in the autoradiographs than did the rest of the brain. In the cerebellum, the granular layer thus seemed to house considerable amounts of radiosulphate (Fig. 1). Blood, blood vessels connective tissue, meninges and chorioid plexus also gave a strong blackening in the autoradiographs.

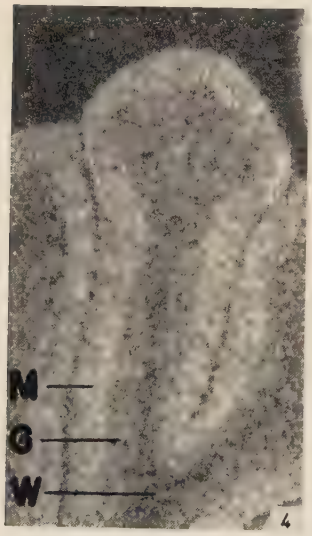
In brains from animals killed 24–192 hours after injection, the average radiosulphate concentration had decreased and considerable changes in the isotope distribution had occurred. The radiosulphate concentration in layers rich in cell nuclei, e.g. the granular layer of cerebellum, had decreased. The surrounding grey and white matter now gave a stronger blackening than the granular layer (Fig. 2). 192–768 hours after isotope injection the radiosulphate concentration in both grey and white matter had decreased. The decrease appeared to be more rapid in grey matter than in white (Fig. 3).

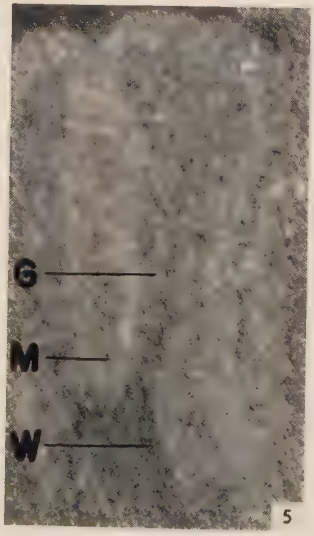
Sections treated with alcohols and xylol gave autoradiographic pictures different from those of the frozen sections. The general diffuse blackening in the autoradiographs of frozen sections 1.5–6 hours after injection and also the high uptake of layers rich in cell nuclei was diminished by the extraction. Insoluble radiosulphate in grey matter could be demonstrated already 1.5–6 hours after injection. The amount of insoluble isotope retained in grey matter increased during 1.5–6 hours, a maximum being attained by 12–48 hours. After 48 hours the uptake in the grey matter seemed to decrease. In animals killed after 24 hours the most significant change that was brought about by the extraction was the removal of almost all radioactivity in the white matter (Figs. 4 and 5). High activities were also found in the meninges and chorioid plexus of animals killed 48 hours and later after injection.











Figs. 1–5. Autoradiographs from cerebellum. Light areas denote high radiosulphate concentration. The densities of the different figures are not directly comparable.
Fig. 1. Frozen section, 1.5 hours after injection. Molecular layer (*M*), granular layer (*G*) and white matter (*W*).
Fig. 2. Frozen section, 24 hours after injection.
Fig. 3. Frozen section, 192 hours after injection.
Fig. 4. Alcohol–xylol treated section, 48 hours after injection.
Fig. 5. Alcohol–xylol treated section, 192 hours after injection.

The alcohol xylene insoluble radiosulphate uptake and the distribution of PAS staining material exhibited a certain parallelism. After alcohol xylol extraction grey matter gave a stronger blackening and also showed stronger PAS-reaction than did white. The distribution of the PAS-reaction appeared to be in general accordance with the distribution reported earlier in the literature [9, 11]. Chorioid plexus and meninges also exhibited a positive PAS-reaction. No certain correlation could be found between the occurrence of metachromasia and radiosulphate uptake in brain tissue.

Discussion. From the uptake curves it appears that radiosulphate was retained by brain tissue. If it is assumed that all activity in the brain at 1.5 hours is due to the presence of blood sulphate, the concentration of isotope in brain tissue exclusive

blood at the other intervals can be calculated. It is found that the maximum for retained sulphate would occur at 12 hours after injection. If on the other hand no activity in the brain were due to radiosulphate in blood the maximum would occur at 3 hours after injection. Thus the maximum concentration of radiosulphate in brain tissue other than blood must be considered to occur between 3 and 12 hours after injection. The curves cannot be taken as altogether representative for nervous tissue as some connective tissue, blood vessels, meninges and chorioid plexus are always present. However, their contribution to the entire activity recorded is inconsiderable.

The autoradiographs revealed that considerable changes in the distribution of the isotope occurred in course of time. An initial accumulation of radiosulphate in layers rich in cell nuclei such as the granular layer of cerebellum was found. The resolution attained with the autoradiographic technique employed, however, does not permit the conclusion that the radiosulphate was present in cell nuclei.

Radiosulphate was found to be retained in white and grey matter. The radiosulphate retained in white matter could be removed with alcohol–xylol extraction. As myelin was removed with this procedure it seems reasonable to assume that the radiosulphate had been incorporated into myelin. Blix [1] has described the presence of sulpholipids in human brain. Recently Holmgård [10] reported that radiosulphate is incorporated into sulpholipids isolated according to Blix. The problem needs further investigation but it seems possible that part of the isotope retained in myelin is present in sulpholipids. The extraction–autoradiography procedure described above can probably be developed into a histochemical method for demonstrating sulpholipids.

Part of the radiosulphate incorporated into grey matter was insoluble with the alcohol–xylol treatment employed. This is in agreement with the results of Boström and Odeblad [4]. A certain parallelism was noted between the alcohol–xylol insoluble isotope uptake and the distribution of the PAS-reaction. Hess [9] has postulated the existence of a polysaccharide containing ground substance within the central nervous system on the basis of studies with the PAS-reaction. The PAS-reacting material has tentatively been classified as a neutral polysaccharide. The existence of a ground substance in the central nervous system has recently been indicated also by electron microscopical work [5]. However, further chemical and morphological evidence is desirable before the existence of a polysaccharide containing ground substance in the C.N.S. can be fully accepted.

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SOME OBSERVATIONS UPON THE DEVELOPMENT AND MODE OF ATTACHMENT OF THE FLAGELLA IN *VIBRIO* AND *SPIRILLUM* SPECIES

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Although some controversy has existed upon the nature and development of bacterial flagella, upon the presence or absence of a blepharoplast, and upon the differences which may exist between the flagella of spirilla and of other bacteria, the majority of workers are in agreement with the classical view, which need not here be restated.

The existence of the blepharoplast has been demonstrated [6, 3, 7, 4], and the growth of flagella through the cell wall has also been proved [3, 6]. It has been claimed that the flagella of spirilla are compound structures, intermediate in some respects between those of protozoa and of other bacteria [4], and it has been suggested that this character is indicative of a primitive philogeny [2].

In the course of a study upon the developmental cycles of spiral bacteria, which will be the subject of a separate communication, a number of observations were made upon these and related points concerning the nature and development of their flagella, and these are the subject of the present paper.

Methods. —Twelve strains of bacteria of spiral morphology were isolated by adding approximately 0.2 per cent of 'Difco' peptone to 25 ml of pond water in a petri dish. This was left at room temperature (25° approx.) for 48 hr. and the surface layer removed by a pipette, transferred to a sterile atomiser and sprayed over plates of the sodium acetate medium described by Pringsheim and Robinow [8]. Colonies recognisable under the plate microscope as consisting of spirilla were picked off, and sub-cultured on the same medium.

Observations.—In certain of the electron micrographs of a saprophytic vibrio the attachment of the single, polar flagellum was clearly visible (Fig. 1). The blepharoplast was embedded in the surface layers of cytoplasm, and was notably small in size, by comparison with those to which the complexes of flagellar fibrils of the larger spirilla were attached. These latter were also clearly visible in certain electron micrographs (Figs. 2, 3). The respective appearances, both of flagella and of blepharoplasts confirmed, in almost every respect the observations of Grace [4]. In those

Fig. 1. Vibrio showing attachment of the single polar flagellum. × 15,400.
Fig. 2. Spirillum with complexes of flagellar fibrils arising from a blepharoplast. The cell wall has a striated macromolecular structure. × 15,400.
Fig. 3. Spirillum with complexes of flagellar fibrils arising from a blepharoplast and with flagellar fibrils in sub-terminal positions. × 7700.
Fig. 4. Gonidia with a single flagella attached to blepharoplasts. × 4600.
Fig. 5. Part of a gonidium with a single flagellum attached to a blepharoplast. × 9200.
Fig. 6. Free gonidial blepharoplasts with attached flagellum. × 15,400.
Fig. 7. Free spirillar blepharoplast with attached bundle of flagellar fibrils. × 11,500.

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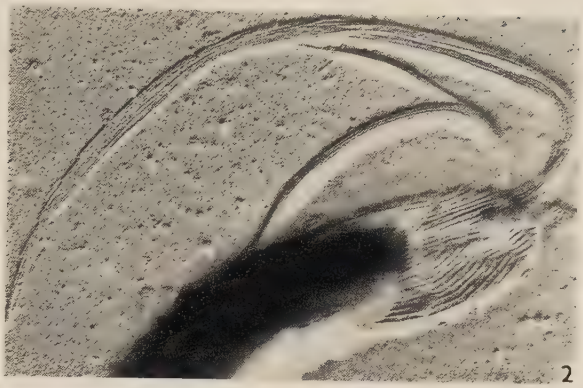
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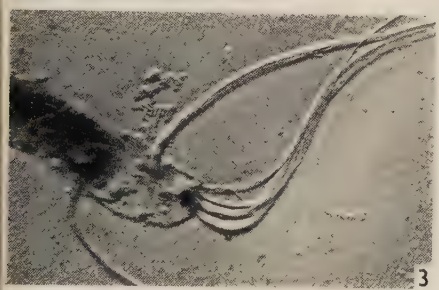
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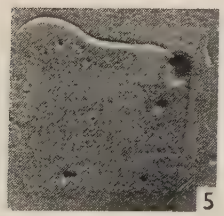
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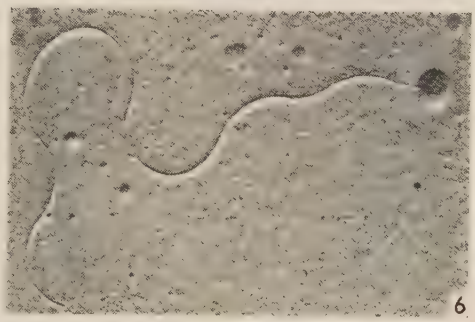
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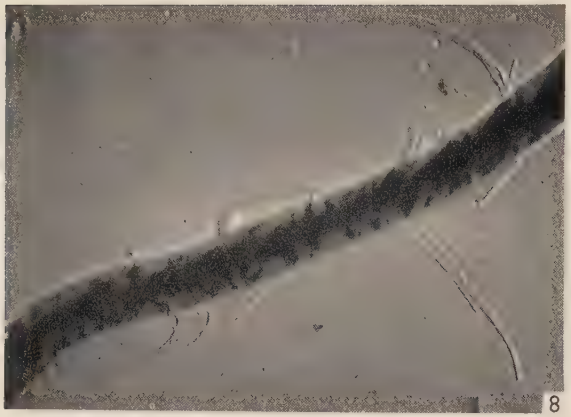

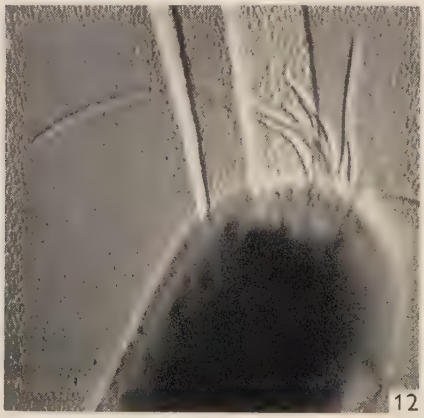

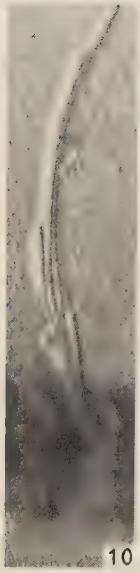

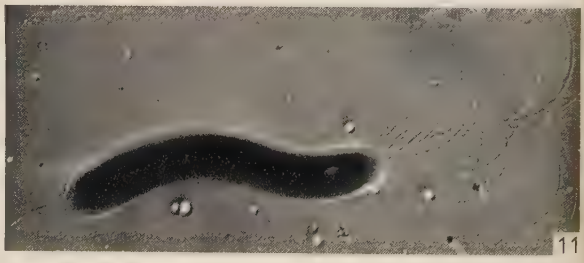

Fig. 8. Spirillum with bundles of flagellar fibrils in lateral positions. × 5800.
Fig. 9. Spirillum with bundles of flagellar fibrils in terminal and lateral positions. × 3100.
Fig. 10. Enlargement of part of Fig. 9, showing a bundle of flagellar fibrils arising within the cytoplasm. × 19,200.
Fig. 11. Spirillum with fully developed flagellar bundle at one pole, and a few short flagella at the other. × 5400.
Fig. 12. Spirillum with a few short flagella at the newly divided pole. × 23,100.

spirilla which underwent a developmental cycle including relatively tiny, motile gonidia, the blepharoplasts of these gonidia were exceptionally clearly visible (Figs. 4, 5), and in the debris of disrupted cells, the blepharoplasts with attached flagella were even found occasionally to be lying free (Figs. 6, 7).

Two further points may be discerned in the electron micrographs of fully developed *Spirillum undula*; one was the striated, macromolecular structure of the cell wall (cf. Houwink [5]) (Figs. 2, 3), and the occasional appearance of tufts of flagellar fibrils (i.e. small, compound flagella) in sub-terminal and lateral positions (Fig. 3). This phenomenon frequently preceded the fission of exceptionally long spirilla (Figs. 8, 9, 10). The newly divided daughter spirilla were thus provided with a full quota of flagella. But in the case of young, rapidly dividing cultures, the condition described by Bisset [1] in dividing pseudomonads obtained; and the daughter spirilla could be observed with one fully developed flagellar bundle, at one pole, and the other, at the newly divided pole, represented only by a few, short bristles (Figs. 11, 12).

Discussion. Evidence is provided by means of electron micrographs of species of *Spirillum* and *Vibrio* to verify the existence of the bacterial blepharoplast, and the development of bacterial flagella by growth through the cell wall. Observations upon the apparent macromolecular structure of the cell wall of *Spirillum* are also included.

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ELECTRON MICROSCOPIC STUDY OF BRONCHIOLAR EPITHELIUM OF NORMAL MOUSE LUNG[1]

PRELIMINARY REPORT

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Although studies dealing with upper respiratory [1, 2, 4, 7, 11] and with bronchiolar epithelium [12] have been reported, a thorough electron microscopic study of bronchiolar epithelium has not been made. In the present work the methods described in

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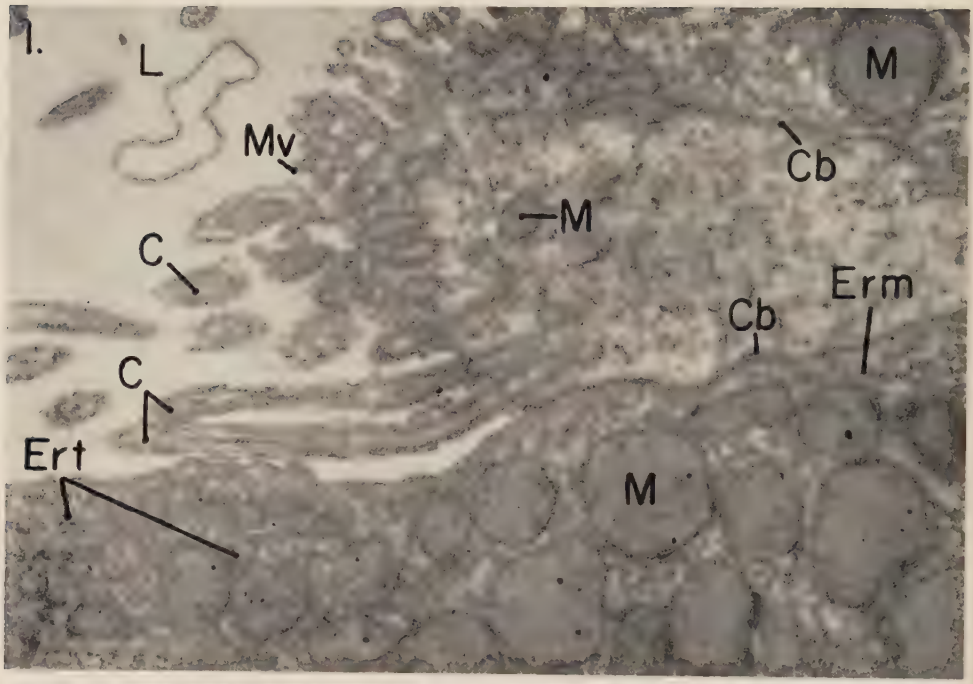
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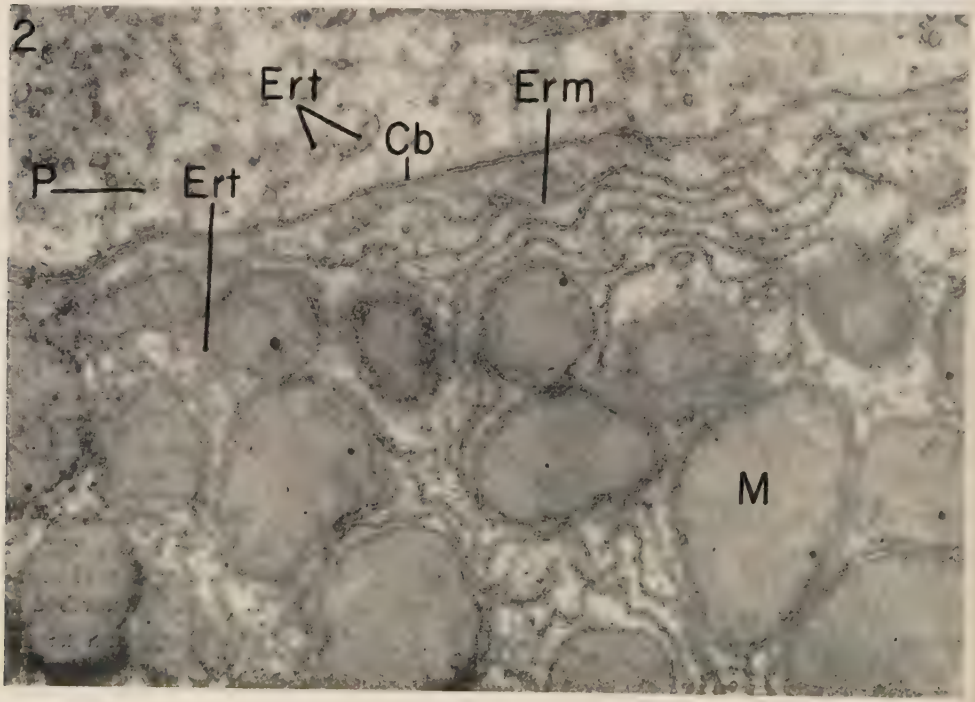
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detail elsewhere [8] have been followed. In order to locate bronchioles, thick sections were taken by hand with a razor blade, using binoculars (30 ×). Often the bronchioles could be recognized on the cut surface of the embedded tissue. Sometimes the unstained sections were examined optically at low power, and the bronchioles were located. The embedded tissue was then trimmed in such a manner that all tissue except a small area of bronchiolar wall was removed.

In the electron microscope, ciliated and non-ciliated cells are recognized (Fig. 1), the latter being the more numerous. The cytoplasm of ciliated cells contains small (60–75 mμ) vacuole-like structures (Fig. 2) which in some micrographs are recognized as probably belonging to the endoplasmic reticulum, thus representing the vesicle-shaped portions (cysternae) of this organelle [10]. Spaced at random, clusters of small (about 150 Å) particles [9] are seen in the cytoplasm of ciliated (Fig. 2) as well as of non-ciliated cells (Fig. 4). Ciliated cells contain relatively few mitochondria, which are mostly located close to the bases of cilia (Fig. 1). The typical mammalian ciliar structure [5] can be seen, in which cross striated rootlets as occurring in lower forms are absent. Microvilli [3] protrude from the ciliated cell surface at irregular intervals [1, 2, 4] (Fig. 1). Their width is 75–100 mμ; their length has not been determined because they are nearly always obliquely sectioned.

The cytoplasmic structure of non-ciliated cells is altogether different from that of the ciliated cells. There is a striking abundance of mitochondria and endoplasmic reticulum (Figs. 1, 2). The mitochondria are usually largest towards the cell base (up to 1μ diameter). They seem to be of spherical shape, as is suggested by their uniformly round or oval form and by the frequent occurrence of tangential sections. Their homogeneous matrix contains the dense, small (25–30 mμ) spherical bodies which were recognized to be a constituant of mitochondria [13] (Fig. 2, 4). Their internal lamellae are not numerous, and do not appear to be radially arranged. Instead they seem to follow a tangential course, as is suggested by their greater abundance in tangential sections through mitochondria (Fig. 2). A second type of mitochondrion, occurring in the same cells, is more slender and elongated, and has numerous parallel internal cristae (Fig. 3). This type is thus comparable to forms recognized in kidney epithelia [13].

The endoplasmic reticulum of non-ciliated cells appears in two forms. It either consists of parallel paired membranes that are often concentric with the large mitochondria (Fig. 2). Or it may also appear in a tubular or vesiculated form [10], either localized in certain (often apical) cell areas (Fig. 3), or intermingled with the double membranes (Fig. 2). Both types occur in one and the same cell (Fig. 3). There is evidence for transitions from one type into the other.

The small particulate component [9] (Fig. 4) in the cytoplasm of non-ciliated cells

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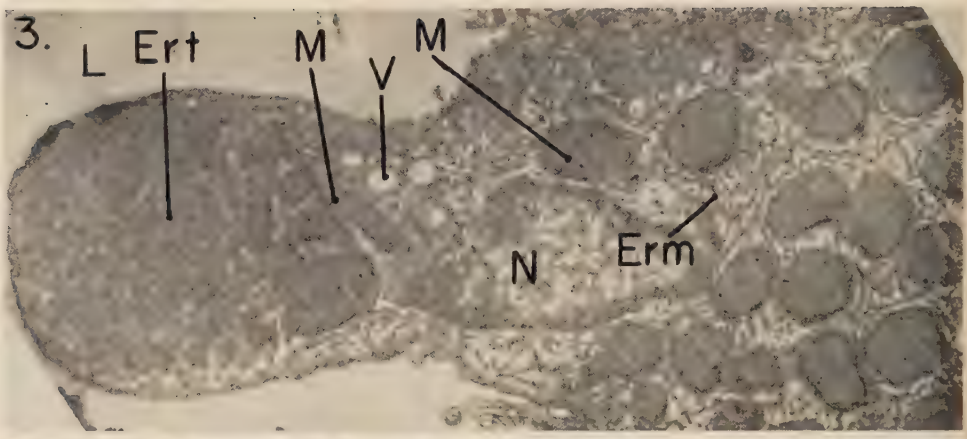
Abbreviations: *C*, cilium; *Cb*, border between two cells; *Erm*, endoplasmic reticulum, double membrane type; *Ert*, endoplasmic reticulum, tubular type; *L*, bronchiolar lumen; *M*, mitochondrion; *Mv*, microvillus; *N*, nucleus; *P*, particulate component of cytoplasm; *V*, vacuole.

Fig. 1. A ciliated cell between two non-ciliated cells. The endoplasmic reticulum of the non-ciliated cell is mostly tubular. Compare with Fig. 2. × 19,000.

Fig. 2. Ciliated (above) and non-ciliated cell. The figure represents the continuation of Fig. 1 towards the right. At the extreme left the reticulum is still mainly tubular as is obvious from the round and oval cross sections. At the right the paired double membranes are predominant. Three tangential sections through mitochondria are evident. × 27,000.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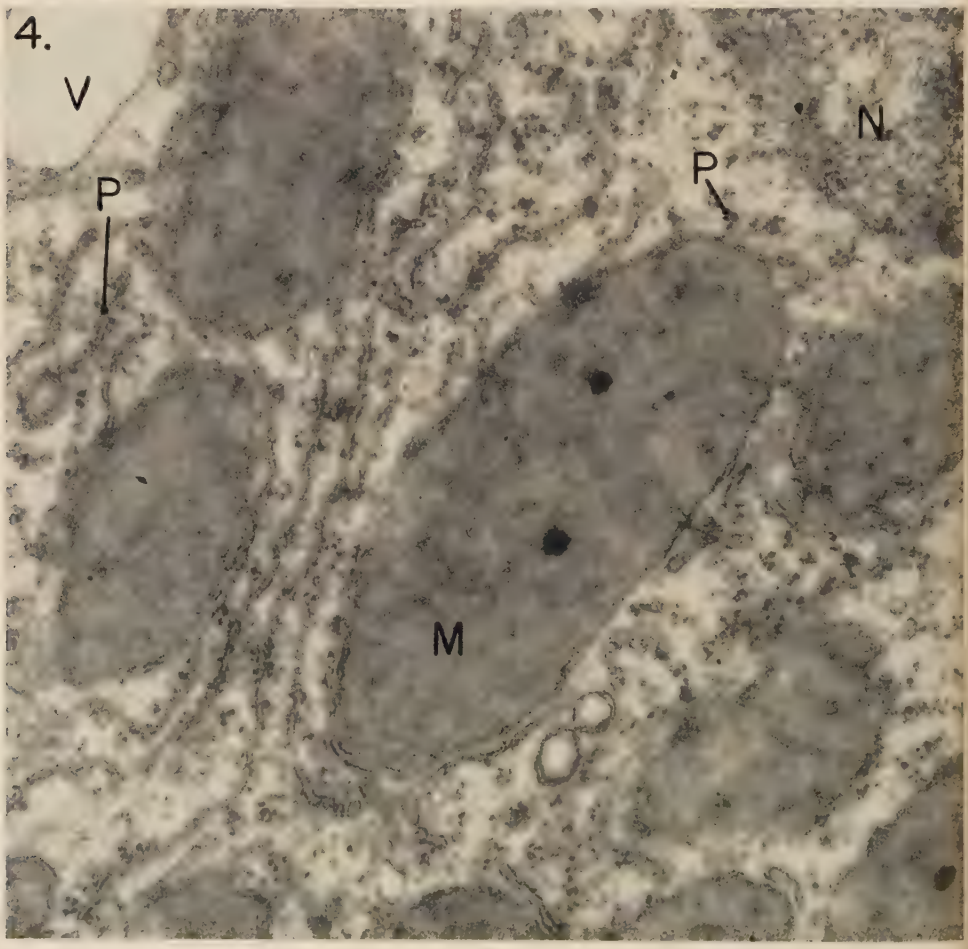
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is in some places closely associated with the membranous type of reticulum [9], but more often there seems to be no relation between the two. A few rather large clear vacuoles are commonly seen (Figs. 3, 4); they obviously are neither related to the reticulum nor to the structures that have been interpreted as Golgi substance [14].

The adjacent surface membranes of two adjacent cells are separated by about the same distance (150 Å) as are the paired parallel membranes of the reticulum (Fig. 2). In spite of suggestions that the latter may be generated at the cell surface [6] a morphological relation between parallelled double membranes and cell surfaces could not be established in our work.

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Fig. 3. A non-ciliated cell protruding into the lumen, showing two types of mitochondria and eticulum. Elongated slender mitochondria and an extensive system of tubular reticulum at left, pherical mitochondria and predominantly double membranes at right. × 13,000.
Fig. 4. Detail from a non-ciliated cell, showing predominantly the double membrane type of eticulum and the particulate component. Note the very low number of internal mitochondrial amellae. × 72,000.

DEPENDENCE OF *ENTAMOEBA HISTOLYTICA* UPON ASSOCIATED STREPTOBACILLUS FOR METABOLISM OF GLUCOSE[1]

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Entamoeba histolytica has long been an enigma to cytochemists and microbiologists. Very little is known concerning its metabolism, its cytochemistry, and its specific growth requirements. This amoeba can be cultured only in the presence of associated

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organisms and very little is known concerning the contribution of the associated organism to the growth of *E. histolytica*.

Reports concerning the utilization of glucose by the amoeba are conflicting. The discrepancies may be due to a number of factors. The first among these may be incomplete removal of the associated organism, and insufficient evidence that these organisms are removed in the washing process. There always remains the possibility that the associated organism may be contributing to some of the data obtained [2]. Secondly, where C^{14}-labeled compounds have been used, the level of C^{14} activity reported as significant has, in closer examination, proved to represent only a few counts above background. Interpretation of such data is difficult [2].

Thirdly, there are strain differences between the amoebae. Differences in their metabolism could account for some discrepancies in results, although so far this has not been demonstrated. Fourthly, the type of organism associated with the amoeba has varied. The metabolism of the associate cannot be ignored in considering the metabolism of the amoeba, since it conditions the environment of the amoeba. An amoeba growing in association with *Clostridium welchii* has a different environment from an amoeba growing with "streptobacilli" and its metabolism may therefore differ, even though both amoebae are of the F-22 strain. Until all of these factors are understood and controlled, the interpretation of any particular set of data may be questionable.

The authors have demonstrated the presence of acid polysaccharides in the cytoplasm and nucleus of both F-22 and K-9 strains of amoebae grown with mono-associated organisms [5]. Variations in acid polysaccharide content appeared to be influenced more by variation in the associated organism than by differences in the strain of amoeba. This was true also of changes in the nature of the plasmolemma (outer limiting surface coat) of *E. histolytica*.

Because the acid polysaccharides in the cell were of the hyaluronic acid type it was decided to determine whether aminosugars were present in the cell, and if so, to what extent. Further, a study of aminosugars might elucidate some of the problems concerning glucose metabolism by the amoeba: whether it occurs, and if so, how and to what extent.

Materials and methods.—For this study, the F-22 strain of *E. histolytica*[1] was grown with penicillin-inhibited streptobacilli in Shaffer-Frye medium [8]. This particular culture was used because the amoebae grow luxuriantly. Prior to inhibition, the streptobacilli were allowed to grow actively for 24 hours. The medium was then centrifuged and the supernatant fluid removed. Penicillin was added to this fluid to inhibit the remaining streptobacilli, and the amoebae were then added. This method of culture results in a two phase system. One phase contains the products of actively metabolizing streptobacilli, while the second contains also penicillin-inhibited streptobacilli. The two phases can be separated by time.

The first part of the experiment was designed to determine whether aminosugars were present, and if so, in what quantities. For this determination, the mucopolysaccharides were hydrolyzed and the concentration of cell hydrolysates of *E. histolytica* detected by the Elson-Morgan procedure as modified by Blix [3] and Boas [4] (see Table I).

[1] Supplied through the courtesy of Dr. Jas. G. Shaffer, Chicago Medical School, Chicago, Ill., U.S.A.

TABLE I

Hexasamine concentration of *E. histolytica.**

No. of amoebae	Elson-Morgan procedure	
	Blix modification μg hexosamine†	Boas modification μg hexosamine†
2.5×10^6	112	
2.4×10^6	104	
2.3×10^6		105
1.8×10^6		117

ig. 1. The graph demonstrates that glucosamine hydrochloride can be separated from other losely allied compounds present in *E. histolytica*. The liquid chromatogram principle was applied, nd the effluent collected in fractions of equal size (0.8 ml) and analyzed. A column of 47.0 × 0.7 m was used to establish the standard. This same column, after adequate washing with 0.3*N* HCl was used to separate the glucosamine hydrochloride (unknown) from the amoebae. Ten 0-ml Shaffer-Frye cultures of *E. histolytica* were concentrated and washed. These contained pproximately 8,800,000 amoebae. They were lyophilized and redissolved in 1 ml of 0.3*N* HCl, alf of which was run through the column.

TABLE II

Radioactivity in *E. histolytica* grown in association with streptobacillus supplied with C^{14} glucose for 24 hours previously.*

Fraction tested	Experiment 1		Experiment 2	
	Counts/min.	% radioactivity	Counts/min.	% radioactivity
Streptobacillus supernatant	48,720	100	228,240	100
Amoeba hydrolysate . . .	11,790	24.2	1,890	0.8
Glucosamine fraction of hydrolysate	1,006	2.06	384	0.12

* See procedure *A*.

TABLE III

Radioactivity in *E. histolytica* grown in association with streptobacillus supplied with C^{12} glucose for 24 hours before C^{14} glucose added.*

Fraction tested	Experiment 1		Experiment 2	
	Counts/mln.	% radioactivity	Counts/min.	% radioactivity
C^{14} glucose	307,215	100	61,443	100
Amoeba hydrolysate . .	665	0,216	0	0
Glucosamine fraction of hydrolysate	0	0	0	0

* See procedure *B*.

The second part of the experiment was concerned with the identification of the particular aminosugar giving the Elson-Morgan reaction. By using a modification of the method of Gardell [7], the aminosugar was identified as glucosamine. Fig. 1 indicates the comparative identity of the aminosugar fraction from the amoeba with the glucosamine standard used.

Becker and Day [1] found that in the rat, glucosamine is derived from glucose without cleavage of the glucose molecule. The third part of the present experiment was designed to determine whether the amoebae utilized glucose or were dependent on the actively metabolizing streptobacilli for its conversion into glucosamine. In procedure *A*, uniformly labeled C^{14} glucose was added to the streptobacilli, and they were allowed to metabolize actively for 24 hours before inhibition with penicillin and the addition of amoebae. In procedure *B*, unlabeled C^{12} glucose was used for the streptobacillus culture, and C^{14} glucose was added 24 hours later with the amoebae, after inhibition with penicillin. In both cases, the amoebae were analyzed for radioactivity after growing for 48 hours.

Results and comment.—As shown in Tables II and III, *E. histolytica* did not utilize glucose. C^{14} was found in the glucosamine fraction only when the labeled glucose was added to actively metabolizing streptobacilli; not when it was added after inhibition of the bacteria. It would appear that the amoebae are dependent upon the actively metabolizing streptobacilli and their ability to convert glucose to glucosamine, which the amoebae then utilize. Analysis of the fluid in which streptobacilli had been grown, after filtering out the bacilli with a Seitz filter, revealed that it contained 0.1 mg/ml of free glucosamine. Therefore glucosamine is freely available to the amoebae in this solution. It is also possible that some of the glucosamine in the amoebae could have come from within the bacilli. However, any glucosamine within the streptobacilli would have to have been present prior ot their inhibition by penicillin. The most likely source would seem to be the soluble products of bacterial metabolism. The Shaffer-Frye medium *per se* contains no glucosamine, free or bound.

In addition, we have been able to maintain cultures of amoebae containing glucosamine 5.0 mg/ml (tc which no associated organism was added after the first transfer), for fifteen transfers (replicate cultures) over eight weeks. Sterility tests performed at appropriate intervals showed no bacterial growth after the second transfer. Penicillinase was added, and each test was watched for two weeks. There was no growth in control cultures containing glucose and no glucosamine. However, the amoebae did not multiply as vigorously in the latter as in Shaffer-Frye cultures, indicating that there are factors in addition to glucosamine which contribute to growth.

It would appear that efforts to demonstrate strain differences between the amoebae [6] are not likely to succeed unless the metabolic activity of the associated organisms is taken into account.

Summary.—*E. histolytica* (F-22, streptobacillus associate) contains relatively large amounts of glucosamine. The amoeba cannot synthesize glucosamine, but is dependent upon the bacterial associate for its supply of this aminosugar. This amoeba does not utilize glucose as such, but only the products of glucose metabolism of the mono-associate. A definite metabolic link is believed to occur between this amoeba and its associated organism.

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ON THE QUESTION OF THE DNA CONTENT OF SEA URCHIN EGGS

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Burgos has recently published some observations which he interprets as indicating that the pronucleus of the mature *Arbacia punctulata* egg is Feulgen positive and hence contains DNA [1]. He states that the results of his experiments disprove the conclusion reached by Marshak and Marshak that DNA is absent from the pronuclei of echinoderm eggs. He has misconstrued the nature of the evidence we have presented and we feel therefore that it is necessary to correct this impression. The misapprehension is stated twice. (1) "One of their most important pieces of evidence is that DNA as demonstrated by the Feulgen nucleal reaction is apparently absent from the nucleus in mature unfertilized sea urchin eggs." (2) "Recently Marshak and Fager [2] and Marshak and Marshak [4] reported that there was an absence of DNA as revealed by the Feulgen reaction in mature unfertilized eggs."

Marshak and Fager [2] reported that the Feulgen reaction in the *Arbacia* pronucleus was negative, but did not claim that this constituted evidence of the absence of DNA for the obvious reason that a negative staining reaction may be attributed to some interference with the reaction as well as to a possible absence of the compound which may be so stained. Burgos erroneously cites this paper as claiming to present proof that DNA is absent from the *Arbacia* pronucleus [1]. In two subsequent papers cited by Burgos [3, 4] data were given on quantitative analyses for thymine in 2 species of echinoderm eggs, in one of which, *Tripneustes*, the acid soluble fraction had been removed and in the other, *Arbacia*, not. In the case of *Tripneustes*, all of the thymine found could be accounted for by DNA of contaminating somatic cells and polar bodies, while with *Arbacia* there was an excess which could be attributed to the egg itself. Analysis of the acid soluble fraction from the *Arbacia* eggs showed that it contained thymine in an amount sufficient to account for all of the excess of total thymine which could not be assigned to non-ovular DNA [5]. Thus in both species none of the thymine found could be justifiably attributed to the egg-DNA. Furthermore it was pointed out that if an amount of DNA indicated by the excess thymine found were to be arbitrarily assigned to the pronucleus it should give a detectible Feulgen reaction if there was no interference. In brief, then, the quantitative chemical analyses together with the microscopic determination of possible sources of DNA in the preparations other than the ova, and not the nature of the Feulgen reaction of the pronucleus, was taken as evidence of the absence of DNA in the ova. In one of our papers cited by Burgos, this was stated very clearly: "In our experiments, neither the isotope data nor the cytological observations alone are sufficient evidence for excluding DNA as the primary genic substance, but as we have tried to show, when both are taken together, this is a necessary conclusion" [4].

Apart from this matter, it is necessary to consider by itself the nature of the evidence that supports the thesis that the *Arbacia* pronucleus is in fact Feulgen positive. Burgos confirms our observation that after thorough lipid extraction the nucleus gives a negative Feulgen reaction, but argues that the procedures used for extracting the lipids produce the negative reaction presumably by removal of the DNA. He neglects to consider the series of controls set up to check just this point. When a mixture of fertilized and unfertilized eggs were carried through the extraction procedure and then subjected to the Feulgen procedure, it was found that the polar body nuclei, the chromosomes of the first meiotic division, the meiotic interphase nucleus, and nuclei of the first cleavage division gave a positive reaction while the pronuclei were consistently negative. This was also found to be the case for *Tripneustes* eggs which were fixed in 70 per cent alcohol and then subjected to lipid extraction. (This egg contains a carotinoid pigment but very little or no echinochrome [Marshak, unpublished observation].) When the lipid was not removed before staining by the Feulgen method, the cytoplasm and all of its granules were found to be red and the pronucleus, at least in its periphery, a darker red. The two illustrations of pronuclei shown by Burgos both have this appearance, i.e., the plasmal reaction has not been eliminated and the color observed is therefore of doubtful significance. Figs. 2 and 4 which apparently show an absence of the plasmal as far as may be judged from the reproduction, do not however show a pronucleus. Thus the most critical piece of evidence needed for establishing his thesis is not illustrated, although other sundry observations are. Although he found that after treatment with DNAse, nuclei were not stained, an essential control, incubation without added enzyme, was omitted. The preparation, although fixed, might nevertheless have contained significant amounts of enzyme other than the DNAse added, e.g. lipase, which could account for the loss in stainability. It would also be good to know and was not mentioned by the author, whether the plasmal as well as the purported nucleal reaction disappeared on incubation with and without the enzyme. Burgos describes Feulgen positive granules deposited "inside" the nuclear membrane of both non-centrifuged and centrifuged eggs. We did not find Feulgen positive material in either type of nucleus [4]. These nuclei do show chromatin strands of the usual thread-like shape after hematoxylin staining [6], and also in unfixed preparations viewed by phase contrast [5]. It therefore seems questionable whether the granules described by Burgos are chromatin and the red color he observed represented DNA.

Reviewing all of the evidence, we see no justification for altering our conclusion that DNA is absent from the mature echinoderm eggs of the species we have examined, nor the deduction that DNA cannot be the sole or the primary gene component.

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THE EFFECT OF LIGHT ON THE MANIFESTATION OF THE ELECTRORETINOGRAM AND ON HISTOCHEMICALLY DEMONSTRABLE SH-GROUPS IN THE RETINA

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In order to study the effect of light on the manifestation and development of the electroretinogram (ERG), an investigation was earlier made of the ERG in newborn kittens [6]. It was found that, in animals reared entirely in the dark, the appearance of the potential could be delayed up to the age of four weeks. In a control group reared in light, a potential appeared before two weeks of age. When the animals reared in the dark were exposed to a light stimulation, that necessary for recording e.g. an ERG (about $^1/_{10}$ sec. flashes, 20 to 30 times over a period of 10 to 20 minutes), an ERG potential could be registered on the following day. This potential was then of the same magnitude as in an animal reared in the light. In reference to histochemical studies by Sidman and Wislocki [5] of the postnatal development of the retina, it has been suggested [6] that there might exist a correlation between variations in the level of SH-groups and those in the functional state of the retina, previously shown to occur under different experimental conditions.

The above ERG studies have thus been extended to include simultaneous histochemical determinations of SH-groups.

Methods.—Nineteen kittens were reared entirely in darkness. The mothers received a daily addition to their diet of 400 I.U. of vitamin D and 1000 I.U. of vitamin A. In 8 of the kittens, the ERG was recorded with Karpe's method [4]. For technical reasons, however, the indifferent electrode was placed in the ear. Before examination the animals were anaesthetized by intraperitoneal injection of about 35 mg of nembutal/kg body weight. The light intensity was 800 lux, since this was earlier found to give maximum recordings in kittens [6]. The animals in which the ERG was not recorded were anaesthetized in the dark and the eyes removed in dark red illumination; during fixation the eyeballs were kept in the dark.

Ten kittens were reared in the light, after which the ERG was recorded and the eyes were removed. All animals were examined at an age of 3 to 24 days. The eyes were fixed unopened in 80 per cent alcohol, containing 5 per cent trichloroacetic acid, for 24 hours. After imbedding in paraffin, they were cut meridionally into sections 10 μ thick. Some of the sections were stained according to Hansen [3] in iron alum hematoxylin and fuchsin picric acid for routine histological study. Sulfhydryl staining was performed according to Bennet's technique [2]. The duration of Red Sulfhydryl Reagent incubation was 12 hours; the specimens were mounted in DPX resinous medium. To test the specificity of the reaction, control sections were pretreated in a saturated solution of phenyl mercuric chloride in alcohol.

TABLE I

Kittens reared in the light.

Day	3	4	5	7	11	12	21	24
pot. in mV	0	0	0	0	0.10	0.13	0.20	0.23

TABLE II

Kittens reared in the dark.

Day	5	9	11	12	12	15	17	19	24
pot. in mV	0	0	0	0	0	0.05	0.05	0	0

Results.—The 8 kittens *reared in the light* exhibited, after the 10th day of life, an ERG of normal magnitude for their age [6], as shown in Table I.

Attempts to record the ERG were made in 9 of the 19 kittens *reared in the dark.* In agreement with earlier investigations [6], a retardation of the manifestation of the ERG was observed. The size of the *b* potential at different ages is shown in Table II.

One of four siblings born in the dark was moved into the light a few hours after birth, and was reared by a foster-mother. On the 24th day, the ERG was recorded in this kitten, as well as in one of those three which had remained in the dark. The recordings are shown in Fig. 1. The eyes of the two remaining kittens were removed without previous ERG recording. The four animals differed in weight by only 5 g, indicating that the nutritional conditions were similar.

A histochemical investigation, but no ERG examination, was made on 10 of the kittens reared in the dark.

Specimens stained according to Hansen [3].—The division into outer and inner nuclear layers was complete by the 7th to 12th day. During the same period the rods lengthened and the outer and inner segments could be distinguished between the 12th and 17th days of life. The differentiation was marked even in the peripheral segments of the retina. No difference could be established between the animals reared in the dark and those reared in the light with respect to these histological criteria of maturity.

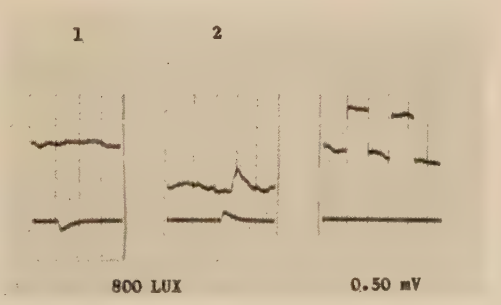
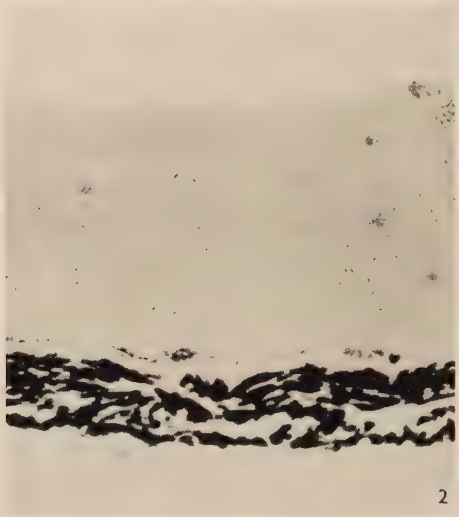


Fig. 1. *1.* Recording in a 24-day-old kitten reared in the dark. No ERG. *2.* Recording from a 24-day-old sibling reared in light. ERG 0.23 mV. To the right: calibration.



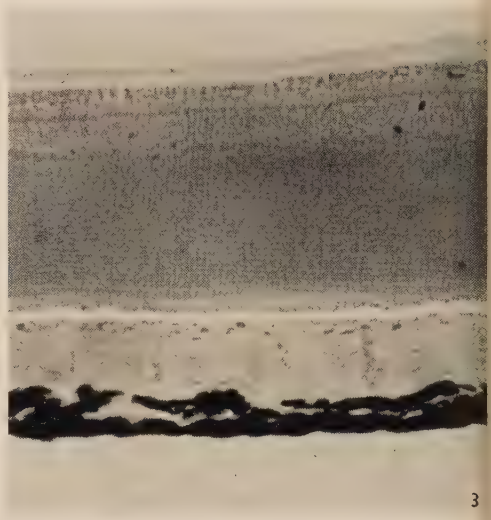


Fig. 2. Eye from a 24-day-old kitten reared in the dark. Bennet's sulfhydryl staining. Extremely weak reaction of the retinal layers. (× 250).
Fig. 3. Eye from a 24-day-old sibling reared in light. Bennet's sulfhydryl staining. The retinal layers, especially the outer nuclear layer, show a fairly intense reaction. (× 250.)

Specimens stained according to Bennet [2]. (i) *Retina.* Irrespective of age, the nuclear layers of the *animals exposed to light* showed a fairly strong positive reaction; after differentiation, the outer layer stained more deeply. The ganglion cells reacted weakly. The plexiform layers and the layer of rods did not react until after about a week, and then only faintly. The *membranae limitantes externa* and *interna* could not be distinguished. During the first days of life, the retinae of the *animals reared in the dark* did not differ from those reared in the light. After a week, a distinct decrease in intensity was observed in the nuclear layers, particularly in the outer one; this became increasingly apparent with advancing age. Thus, in animals aged 21 to 24 days, the outer nuclear layer was stained extremely faintly or not at all. The intrinsically weak staining of the layer of rods was not affected in the animals reared in the dark. The retina of the animals *reared in the dark but exposed to strong light intensity at the ERG examination* exhibited the same features as that of animals reared entirely in the light. Figs. 2 and 3 show SH staining of the retina in two 24-day-old siblings, one reared in the dark (Fig. 2) and the other in the light (Fig. 3).

(ii) *Other structures of the eye.* Irrespective of age and exposure to light, the lens, in particular its central parts, showed a strong positive reaction. The anterior epithelium of the cornea, as well as its endothelium, reacted moderately. The corneal stroma exhibited no reaction. The choroid stained weakly, whereas, generally, the sclera did not stain. No age variations, nor any difference between animals reared in the dark and in the light, could be observed in the aforementioned structures.

The specimens pretreated with phenyl mercuric chloride showed no SH staining.

Discussion.—As most histochemical methods, sulfhydryl staining is a qualitative rather than a quantitative test for localization of the substance in the specimen.

Moreover, certain special limitations seem to be inherent in Bennet's [2] method. The relatively faint colour of the reaction has been pointed out by Barrnett [1]. Despite these difficulties, it can be assumed with some degree of probability that a considerable difference between the intensity of staining in different specimens reflects a difference in the level of sulfhydryl groups.

According to the results obtained with this method of investigation, SH-groups are present at birth in relatively large quantities in the retina of kittens. If the animals are not allowed to be in contact with light during their first weeks of life the level of SH-groups in the retina appears to decrease.

The fact that, in kittens reared in the light, the ERG potential cannot be recorded during about the first 10 days of life, is possibly explained by the immaturity of the retina. Thus, although SH-groups are present in relatively large quantities, this is not a primary prerequisite for the manifestation of the potential.

If kittens are reared in the dark, the appearance of the ERG potential can generally be delayed (although for at most about three weeks), despite the fact that the morphological maturation of the retina proceeds irrespective of deprivation of light. It is under these conditions that a primary correlation between ERG potential and the level of SH-groups can be postulated.

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A NEW APPROACH TO HIGH RESOLUTION MICRORADIOGRAPHY USING EXTREMELY SOFT X-RAYS[1]

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From the physical point of view, the concept of high resolution in microradiography is of the greatest importance, since the resultant preparation is of microscopic dimensions and must be enlarged tremendously for observation. In order to attain extremely high resolution of specimens of biological material, several technical requirements

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must be fulfilled, the most important among which is perhaps to obtain a source of very soft X-rays.

It has been shown [4] that a microscopic section of thickness t cm and density ϱ g · cm^{-3} (the water having been removed by dehydration during histological processing) shows a maximum of contrast when the potential V applied to the X-ray source follows the expression

$$V = 1.5 \times 10^4 \cdot t^{\frac{1}{3}} \cdot \varrho^{\frac{1}{3}}.$$

For ordinary sections of thickness ranging between 1 and 5 microns, the voltages which must be applied vary between a few hundred volts up to about 2000 volts. In order to obtain and utilize such extremely soft X-rays in the sample chamber, the window of the X-ray tube must be very thin and the sample chamber must be evacuated. In the equipment especially constructed for the present purpose, the window consisted of a 1000 or 2000 Å thick aluminum foil prepared by evaporation. The 1000 Å thick window transmits about 30 per cent of the X-rays at 50 Å. The chamber containing the sample and emulsion was maintained at high vacuum. Details of the equipment will be published later [4].

Assuming an ideal source of radiation, the largest single factor determining the resolution of the final product is the "interspace", i.e., the distance between specimen and photographic emulsion [2, 3]. We have applied a modification of a technique frequently used in autoradiography the so-called "mounted method" [1]. This method consists of floating the histological section directly on a partly hydrated photographic emulsion. After the emulsion-specimen complex has become dry, the "interspace" is reduced to an almost insignificant value.

In addition to the "interspace value" another important factor in determining the resolution of microradiograms is the size of the developed silver grains in the X-ray sensitive emulsion. Again, it holds especially in this work, since high magnifications are employed for observation. Thus, if the grain size is large, as in ordinary X-ray emulsions, the finer resolution of details would be obscured by graininess. It is fortunate then that essentially grainless X-ray sensitive emulsions, such as that of the Kodak Spectroscopic Plate No. 649 employed in this study, are available to the investigator.

In practice, the following technique was employed for obtaining the microradiograms illustrated in Figs. 1 to 9. All steps were carried out in a photographic darkroom in the light of a Wratten Series 1 (red) safelight. Pieces of Spectroscopic Plate No. 649 of a suitable size for the chamber of the X-ray apparatus (approximately one centimeter square) were dipped in a 2 per cent solution of celloidin in ether-ethanol (1:1) for approximately 30 seconds. The celloidin solution had been filtered

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High resolution microradiograms of successive stages of mitosis in *Allium cepa* root tip. Material fixed in Flemming's (Figs. 3, 4, 5, 6 and 8) or Allen's (Figs. 1, 2, 7 and 9) mixture, embedded in paraffin, and sectioned at 2 microns. The specimens exposed to X-rays of 1.8 kV. Structures possessing a high X-ray absorption appear white in the illustrations.

Fig. 1. Interphase nucleus. Note the high dry weight of the nucleolus. Fig. 2. Early prophase showing beginning spireme formation. Fig. 3. Prophase; Spireme formation is almost complete, and the nuclear membrane is breaking down. Fig. 4. Early metakinesis. Fig. 5. Late metakinesis. Fig. 6. Metaphase, with chromosomes distributed equatorially. Fig. 7. Diakinesis. A beaded structure of the chromosomes is apparent. Fig. 8. Telophase. The new cell wall is forming. Fig. 9. Two daughter cells in early interphase.

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through sintered glass prior to use, to remove particulate material which could interfere with the subsequent X-ray procedure. After dipping, the pieces of plate were suspended in a vertical position to dry for at least 12 hours. The celloidin membrane thus formed over the emulsion was of the order of 1000 Å in thickness. While this increase in "interspace" is probably of significance in adversely affecting the resolution of the X-ray image, it is nonetheless necessary. It serves the two-fold purpose of allowing easy removal of the tissue specimen after exposure to X-rays, and also tends to protect the emulsion from mechanical injury.

Histological sections from paraffin blocks were floated on the surface of filtered distilled water at 37–40°C until they were free of wrinkles. The celloidin-coated pieces of plate were then submerged in the same water, emulsion-side up, for one to two minutes, until the emulsion became somewhat hydrated and swollen. Using a pair of fine-tipped forceps, each plate was slowly lifted out of the water. Simultaneously, using a sable brush, one paraffin section was gently brushed on to the emulsion surface. The resultant tissue-emulsion complex was allowed to dry in a vertical position for 12 to 24 hours.

After drying, the preparations were deparaffinized by immersing them for 2 to 5 minutes in petroleum benzene. During the deparaffinizing, the plates were maintained in the horizontal and only the benzene was agitated. This minimized the possibility of removing the section prematurely. After the plates were removed from the deparaffinizing bath, the adherent benzene evaporated within one minute, and the plates were then ready for exposure to X-rays. A reference system for the quantitative evaluation [2, 3] of the microradiogram must be added at this stage.

Following the exposure, the plates were immersed in ether–ethanol (1:1) for 2 minutes. With moderate agitation, the sections usually became disengaged from the emulsion during the first minute. Any residual material on the emulsion surface was removed by very gently wiping the emulsion with a finger tip moistened in ether–ethanol. The plates were then drained for a few seconds and were then developed according to routine photographic technique.

It has been found possible to transfer the section from the emulsion surface to an albuminized microscope slide for subsequent histological treatment. Thus, it is feasible to mount the stained histological section side-by-side with the microradiogram for microscopic observation and photomicrography.

The plate shows that the ultimate resolution of the procedure outlined above, i.e. the combined use of extremely soft X-rays (200–2000 volts) with the "mounting technique", is only limited by the resolution of the light microscope used to inspect the microradiograms.

Further experiments to obtain an X-ray resolution better than 0.1 micron are in progress.

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FURTHER OBSERVATIONS ON THE ACTION OF RIBONUCLEASE ON LIVING AMOEBAE

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has been shown [2] that crystallized ribonuclease (G.B.I. 0.1 mg/ml) exerts powerful inhibitory effects on the locomotion and the multiplication of amoebae; the enzyme also produces a marked decrease (20–45 per cent) in the ribonucleic acid content and a considerable inhibition (80–90 per cent) of the incorporation of labelled phenylalanine into the proteins. The addition of yeast ribonucleic acid to the ribonuclease-treated amoebae has distinctly favourable effects. It is the purpose of the present note to give additional results bearing on the mode of action of ribonuclease on living amoebae.

The penetration of ribonuclease into the amoebae is a rapid process, possibly linked to pinocytosis [6]: already 5–10 min. after the addition of the enzyme, basiphily (Unna staining) decreases in many amoebae, especially in their nucleoli. A detectable (20 per cent) drop in the RNA content is also found, with the quantitative method of Caspersson and Brachet [8], after a 15-minute treatment. There is no evidence, in amoebae, for an initial synthesis of ribonucleic acid, as found by Ledoux and Baltus [7] for Ehrlich ascites tumour cells. Finally, estimations of the ribonuclease content of amoebae which have been left in contact for 30 minutes with the enzyme show a 20–65 per cent increase over the controls. These experiments, taken together with the fact already mentioned [2] that radio iodine-labelled ribonuclease is quickly found in the amoebae (including their nucleus), leave no doubt that the enzyme really penetrates inside the cells; the nuclear membrane obviously is no effective protection against further penetration into the nucleus. In this respect, our results agree with those of Anderson on isolated nuclei.

As a control, the effects of different substances have been studied at the 0.1 mg/ml concentration on living amoebae: while basic proteins like histone and salmine are very toxic and quickly kill the amoebae, cytochrome c (which has almost the same molecular weight as ribonuclease) has no effect on basiphily, despite obvious toxicity. Very large molecules, like polymerized desoxyribonucleic acid, also show some toxicity; but it has been impossible to detect any accumulation of desoxyribonucleic acid in the treated amoebae, with either the Feulgen reaction or basic dyes. Crystalline desoxyribonuclease is about 10 times less toxic than ribonuclease; it has no effect on cytoplasmic basiphily; the Feulgen reaction in the nucleus and in the ingested foods is not reduced in intensity. While ribonuclease very strongly inhibits (90 per cent) the incorporation of phenylalanine into the proteins, desoxyribonuclease only exerts a slight inhibitory effect (20 per cent). If this inhibition means penetration of the enzyme into the amoebae, desoxyribonuclease is different from ribonuclease in not attacking its natural substrate inside the cells.

In view of the findings of Lundblad and Hultin [9] and Roth [10] that ribonuclease releases proteolytic enzymes and desoxyribonuclease from homogenates, the possibility exists that protein anabolism is inhibited in the amoebae because ribonuclease liberates an intracellular protease. Such an indirect mechanism can, however, be ruled out for two reasons. First, the amino acids:protein ratio remains constant after a 1–2 hrs. ribonuclease treatment. Secondly, such a treatment does not increase the proteolytic activity of the amoebae (method of Holter and Løvtrup [5]), which, on the contrary, decreases considerably (56–86 per cent inhibition). In agreement with this finding, we noticed that digestion of preys (including their desoxyribonucleic acid) is considerably delayed, if not stopped, when well-fed amoebae are treated with ribonuclease during 1 hour before they are starved.

Experiments in which amoebae were treated with ribonuclease during 1 hour at different temperatures have shown that the effects of the enzyme on shape, motility and basiphily are much stronger at 25° than at 7°.

It can be concluded that ribonuclease really penetrates into the living amoebae; the enzyme then breaks down their cytoplasmic and nucleolar ribonucleic acids. This breakdown, in contrast to the situation found in onion roots [3] begins very soon after the enzyme has got into the amoebae. There is no evidence that ribonuclease acts in an indirect way, in releasing a protease for instance; it is likely that, as in Gale and Folke's [4] experiments on disrupted Staphylococci, the integrity of the ribonucleic acid is necessary for amino acid incorporation into the proteins of the living amoebae.

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